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IDENTIFICATION OF DISEASE-CAUSING GENETIC VARIANTS IN PATIENTS WITH SEVERE EARLY-ONSET IMMUNOLOGICAL DISORDERS: A WHOLE-EXOME SEQUENCING APPROACH

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LIST OF ABBREVIATIONS

ADA	adenosine deaminase
AGS	Aicardi-Goutières syndrome
ANA	antinuclear antibodies
ANCA	antineutrophil cytoplasmic antibodies
APC	antigen presenting cell
ATP	adenosine triphosphate
CANDLE	chronic atypical neutrophilic dermatosis with lipodystrophy and elevated temperature
CAPS	cryopyrin associated periodic syndromes
CECR1	cat eye syndrome chromosome region, candidate 1
CFTR	cystic fibrosis transmembrane conductance regulator
CID	combined immunodeficiency
CLEC16A	C-type lectin domain family 16
CMV	cytomegalovirus
CNS	central nervous system
CNV	copy number variants
COMT	catechol-O-methyltransferase
cPACNS	childhood primary angiitis of the central nervous system
CRP	C-reactive protein
CSF	cerebral spinal fluid
CT	computerized tomography
CTLA4	cytotoxic T-lymphocyte-associated protein 4
CVID	common variable immunodeficiencies
DC	dendritic cell
DNASE1L3	deoxyribonuclease I-like 3
dsDNA	double-stranded DNA
dsRNA	double-stranded RNA
EBV	Epstein-Barr virus
EMSA	electrophoretic mobility shift assay
F2A	FDMV 2A peptide

FCAS	familial cold autoinflammatory syndrome
FDMV	foot-and-mouth disease virus
FOXP3	forkhead box P3
GAF	gamma-interferon activation factor
GAS	gamma-interferon activation site
GvHD	graft-vs-host disease
GWAS	genome-wide association studies
HGCS	human gene connectome server
HHV	human herpes virus
HIV	human immunodeficiency virus
HLA	human leukocyte antigen
HPIV3	human parainfluenza virus type 3
HSCT	hematopoietic stem cell transplantation
HSE	herpes simplex encephalitis
HSP	hereditary spastic paraparesis
HSV	herpes simplex virus
IFIH1	interferon induced with helicase C domain 1
IFN	interferon
IFNAR	anti-IFN-α/β receptor
IgA	immunoglobuline A
IgE	immunoglobulin E
IgG	immunoglobulin G
IgM	immunoglobulin M
IL-1	interleukin-1
IL-6R	IL-6 receptor
iMCD	idiopathic MCD
IPEX	immune dysregulation, polyendocrinopathy, enteropathy, X-linked
iPSC	induced pluripotent stem cell
IRF7	interferon regulatory factor 7
IRGM	immunity-related GTPase M
ISG	IFN-stimulated gene
ISGF3	interferon-stimulated gene factor 3

ISRE	IFN-stimulated response element
IVIG	intravenous immunoglobuline
JAK	Janus kinase
LPS	lipopolysaccharide
mAb	monoclonal antibody
MAVS	mitochondrial antiviral signalling protein
MCD	multicentric Castleman's disease
MeFV	Mediterranean fever
MeV	measles virus
MHC	major histocompatibility complex
MMR	measles-mumps-rubella
MRI	magnetic resonance imaging
MSCV	murine stem cell virus
MWS	Muckle-Wells syndrome
MX1	MX Dynamin-Like GTPase 1
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
NGS	next generation sequencing
NK cell	natural killer cell
NLR	NOD-like receptor
NLRP3	NOD-like receptor (NLR) family, pyrin domain containing 3
NOD2	nucleotide-binding oligomerization domain-containing protein 2
NOMID	neonatal-onset multisystem inflammatory disease
OAS1	2'-5'-oligoadenylate synthetase 1
PAN	polyarteritis nodosa
PBMC	peripheral blood mononuclear cell
PCR	polymerase chain reaction
pDC	plasmacytoid dendritic cell
PHA	phytohemagglutinin
PID	primary immunodeficiency
PKCδ	protein kinase C δ
PMA	phorbol myristate acetate
PME	primary measles encephalitis

PRKCD	protein kinase C, delta
RIG-I	retinoic acid–inducible gene 1
RSV	respiratory syncytial virus
RT-qPCR	real-time quantitative polymerase chain reaction
SAMHD1	SAM domain and HD domain-containing protein 1
SAVI	STING-associated vasculopathy with onset in infancy
SCID	severe combined immunodeficiency
shRNA	small hairpin RNA
sIgAD	selective IgA deficiency
SLE	systemic lupus erythematosus
SMS	Singleton-Merten syndrome
SNV	single nucleotide variant
SSPE	subacute sclerosing panencephalitis
(-)ssRNA	(-) single-stranded RNA
STAT2	signal transducer and activator of transcription 2
STING	stimulator of interferon genes
SV40	Simian virus 40
TCR	T cell receptor
Th	T helper cell
TLR	Toll-like receptor
TNF-α	tumor necrosis factor alpha
TNFAIP3	tumor necrosis factor, alpha-induced protein 3
TRAP	triiodothyronine receptor auxiliary protein
Treg	regulatory T cell
TREX1	three prime repair exonuclease 1
TYK2	tyrosine kinase 2
UCD	unicentric Castleman's disease
UTR	untranslated region
VOD	veno-occlusive disease
VSV	vesicular stomatitis virus
WES	whole-exome sequencing
WGS	whole-genome sequencing

ABSTRACT

The main objective of this project is to identify novel genes capable of causing monogenic defects in the human immune system by studying paediatric patients with severe immunological disorders. While monogenic diseases make up only a small fraction of immunological disorders, they provide insight into mechanistic pathways that are likely to contribute to common forms of disease when less deleterious variants are inherited. Additionally, genetics studies on patients with severe early-onset immune pathologies can increase our understanding of the physiology and functioning of the human immune system. To clarify the contribution of novel genes in severe immunological disorders, the following methodology is used: 1) identification of candidate genetic variants through the use of whole-exome sequencing, 2) detailed assessment of the peripheral immune system in order to gain insight in disease mechanisms and pathways and 3) verification of the role of an identified candidate genetic variant in disease pathogenesis by developing functional tests on a case-by-case basis. This approach was successfully used to identify disease-causing variants in three different genes responsible for severe early-onset immune pathology in four unrelated families with very different clinical presentations:

1. A *de novo* heterozygous gain-of-function mutation in *IFIH1* in a 16-year-old girl with severe early-onset and refractory systemic lupus erythematosus with secondary antiphospholipid syndrome, selective IgA-deficiency and mild lower limb spasticity without neuroradiological manifestations.
2. Recessive homozygous loss-of-function mutations in *CECR1* in a 9-year-old boy with Castleman's-like disease and in two brothers with combined immunodeficiency, lymphoproliferation, autoimmunity and vasculopathy. The success of allogeneic hematopoietic stem cell transplantation both in rescuing the immunologic phenotype and in preventing further vascular disease in ADA2-deficiency was demonstrated.
3. Recessive compound heterozygous loss-of-function mutations in *STAT2* in two siblings with severe viral illness.

INTRODUCTION

Disorders of immunity can give rise to a wide spectrum of diseases. This work focuses on the identification of disease-causing genetic variants in paediatric patients with primary immunodeficiency (PID) and autoimmune/autoinflammatory disease. In the introduction, the rationale for studying the underlying genetic causes of these rare conditions in these specific populations will be explained. Next, a framework for genetic studies in severe early-onset immune pathology will be provided, with an overview of the underlying principles and steps that must be followed in order to prove that a candidate genetic variant is in fact disease-causing. Finally, an overview will be given of the diseases that have been studied in this work.

1. Rationale behind studying the underlying genetic defect in severe paediatric immune pathology

1.1 Clinical impact of PIDs and autoimmune/autoinflammatory diseases

PIDs often present and are diagnosed in infancy or childhood, when a failure to mount an adequate immune response to invading pathogens results in recurrent and/or severe infections [1]. Although PIDs were originally considered to be rare, recent studies estimate that up to 1 in 1200 living people worldwide could be affected by a form of PID [1], indicating that the prevalence of PID could be similar to that of for instance leukaemia [2]. Therefore, PIDs form an important group of diseases in paediatric populations.

In contrast, the majority of patients with autoimmune illnesses develop disease in adulthood [3, 4]. These common forms of autoimmune disease affect approximately 5% of the total world population [3] and are thought to be the consequence of both polygenic and environmental factors [5]. Although disease onset in childhood is much less common [6], it is often associated with increased disease severity and a worse prognosis [3, 7, 8]. Additionally, while common adult-onset (autoimmune) disorders are thought to have a complex multifactorial causation, severe early-onset autoimmune disease is more likely to be monogenic or oligogenic in origin [5]. Similarly, the more recently identified autoinflammatory diseases are rare conditions,

also often present in infancy, are likely monogenic and are associated with a high morbidity and mortality and decreased quality of life [4, 9, 10]. So despite their low prevalence compared to other childhood diseases, early-onset immune diseases are clinically important and severely impact the lives of affected individuals and their families.

1.2 Importance of genetic studies for the diagnosis and treatment of PIDs and autoimmune/autoinflammatory diseases

Treatment of PIDs and autoimmune/autoinflammatory diseases is often accompanied by serious side-effects and can hold risks on its own [11]. Understanding the genetic cause of severe paediatric immune pathologies is critical for the diagnosis and correct treatment of these patients, and for the development of new and better targeted therapeutics. For example, for years treatment of severe early-onset autoinflammatory disease depended on the use of systemic corticosteroids, which was associated with severe side-effects such as growth retardation, osteoporosis, metabolic disorders, muscle atrophy, delayed wound healing or gastrointestinal ulcers. The discovery of for example gain-of-function mutations in the interleukin-1 (IL-1)-regulating gene *NLRP3 (*NOD-like receptor (NLR) family, pyrin domain containing 3) in the cryopyrin associated periodic syndromes (CAPS), a severely debilitating autoinflammatory disease, has led to the successful use of the IL-1 receptor antagonist monoclonal antibody (mAb) anakinra and the anti-IL-1β mAb canakinumab to control this disease [12]. Even when no specific treatment is available, the identification of the underlying genetic defect responsible for their disease is often very important for these patients, as it allows them to label their disease and gain understanding of disease pathogenesis and prognosis.

As mentioned before, severe paediatric immune diseases are more likely to have a monogenetic cause [5] and therefore make up good candidates for genetic studies. Understanding the underlying genetic defect in severe early-onset immune pathology may not only be beneficial for patients suffering from these conditions. Although monogenic diseases make up only a small fraction of immunological disorders, they provide insight into mechanistic pathways that are likely to contribute to common

forms of disease when less deleterious variants are inherited [5, 13]. On that account, use of new therapeutics that were developed for monogenic diseases can be extended to a variety of related immune diseases. For example, discovery of the disease-causing mutations in *NLRP3* that cause CAPS and subsequent research in the disease pathogenesis uncovered the key role of IL-1 in an extended spectrum of autoinflammatory conditions. Subsequently, IL-1 blockade has been successfully used in other autoinflammatory diseases with a known genetic defect (e.g. mevalonate kinase deficiency) as well as in autoinflammatory diseases that are not genetically well defined (e.g. systemic juvenile idiopathic arthritis or Still's disease) [12]. Furthermore, the link between metabolic stress and IL-1β-mediated inflammation through activation of the NLRP3 inflammasome by accumulation of metabolic substrates such as monosodium urate crystals, glucose or cholesterol; provides a rationale for therapeutically targeting IL-1 in prevalent diseases such as gout, diabetes mellitus and coronary artery disease [12]. Clinical trials based on this hypothesis are being conducted at the moment. For now the clinical impact of IL-1 blockade is best established in acute gout, where IL-1 blockade leads to a significant relief of pain, decreased inflammatory markers and a significant reduction of the number of gout flares [12].

1.3 Increasing our understanding of physiology through mechanistic studies of patients with severe early-onset immune pathology

Apart from clinical reasons, genetics studies on patients with severe early-onset immune pathologies are also important for improving our understanding of the physiology and functioning of the human immune system [5, 14]. Studying human inborn errors of immunity can reveal crucial pathways underlying both physiological and pathological processes. For example, identification of a mutation in *MEFV* (Mediterranean fever), encoding pyrin (S242R), in a disease characterized by childhood-onset recurrent episodes of neutrophilic dermatosis and systemic inflammation, revealed a guard-like mechanism of pyrin regulation in humans. The mutation results in the loss of a 14-3-3 binding motif at phosphorylated S242, Similar, loss of both S242 phosphorylation and 14-3-3 binding was observed for bacterial effectors that activate the pyrin inflammasome, indicating that the S242R mutation

thus recapitulates the effect of pathogen sensing, and triggers inflammasome activation and IL-1β production [15]. These studies are complementary to genetic studies in animal models (e.g. murine models), that are valuable because they allow testing of hypotheses under different experimental conditions but are also subjected to certain limitations. A murine phenotype is typically associated with and tested in a single genetic, highly inbred background, making the animals more likely to be homozygous for modifying genetic factors. Human patients are never 100% homozygous, and the identification of a genetic defect in several unrelated individuals with a similar disease phenotype but a different genetic background strongly supports the causal role of the genetic defect in the examined disease. Another limitation lies in the fact that animal models are kept in a tightly controlled environment, which can influence the occurrence of a certain phenotype. In contrast, humans are constantly exposed to a variety of environmental factors which can provoke the occurrence or influence the manifestations of a certain phenotype. Together with interspecies variability these limitations can be responsible for observed differences in phenotypes between animal models and human patients with similar mutations in the same gene.

2. Framework for genetic studies in severe early-onset immune pathology

2.1 Genetics in human immune disease

Until recent years, analysis of the genetic basis for immune pathology has been a slow process of identifying candidate genes based on expression patterns and reverse engineering mutations in these genes in mice to determine the impact on immunity [16], a process called reverse genetics. By contrast, forward genetics is the process of identifying important genes by large-scale genetic analysis of cohorts with strong phenotypes. In immunology forward genetics in mice has proven a tremendous boon [17]. In human patients with a variety of strong phenotypes forward genetics has been used successfully in the search for mutations in familial cohorts of (consanguineous) patients or in large groups of unrelated sporadic cases [18]. Genome-wide linkage analysis and candidate gene approaches have led to the discovery of over 4000 single-gene inborn errors [5] (http://www.omim.org/statistics/entry). However, this approach has proven more difficult in (immune) pathologies without a distinct well-understood

clinical presentation and/or without large consanguineous patient families. Still, severe sporadic immune disorders in non-consanguineous families may also be monogenic in nature and may be caused by familial single-gene defects or by *de novo* mutations. The development and availability of large scale genetic screening methods such as whole-exome sequencing has made it possible to further extend the forward genetic approach to the entire human population. In Belgium for example 10 million people are accessible for immunological screening through medical health services. This represents genetic diversity on a scale inconceivable using mouse models, a resource of tremendous potential to identify genes involved in immune pathology.

2.2 Genetic studies in single patients/families or sporadic cases

Several patients who are followed in the University hospital of Leuven, Belgium, have a possible monogenic disease with a very distinctive phenotype that has not been found or described in other patients. Experts in statistical genetics have formulated their doubts on genetic studies in single patients and emphasize "the critical primacy of robust statistical genetic support for the implication of new genes" [19]. They state that "multiple confirmations of causality should be obtained in multiple unrelated patients" or that "statistical support from multiple cases is strongly encouraged" [19]. Clearly being able to confirm certain gene variants in a group of patients with the same clinical phenotype provides added confidence that the altered gene is responsible for the phenotype. Additionally, it is not possible to firmly conclude full clinical penetrance of a certain genotype found in a single, albeit severely affected, patient; given that modifying genetic, environmental and stochastic factors may be contributing to the phenotype as well. Nevertheless single-patient reports are not uncommon in the field of immunology, where for example 49 out of 232 (21%) examined monogenic PIDs were first reported on the basis of a single case [14]. Although extensive experimental validation of the causal role of a new genetic defect is required in single-patient studies to compensate for the lack of genetic confirmation in unrelated pedigrees, discovery of the causal gene in such a single patient could lead the way for its confirmation in other patients [14].

Criteria have been proposed by experts in the field for establishing a causal relationship between a candidate genotype and the clinical phenotype of a single patient [14]:

1. the candidate genotype must be monogenic, may not occur in healthy individuals and must have a frequency less than or equal to the frequency of the phenotype
2. the genetic variant(s) must impair, destroy, or alter the function of the gene product
3. the causal relationship between the candidate genotype and the clinical phenotype must be established via a relevant cellular or animal phenotype:
    a. for disorders that affect the function of a cell present in the patient: a patient-specific relevant cellular phenotype should be caused by the mutant allele and this phenotype should be corrected by a rescue experiment
    b. for disorders that affect the development of a cell lacking in the patient: development of an animal model that recapitulates both the cellular and whole-organism phenotypes may replace the characterization of a relevant cellular phenotype

A detailed overview of these criteria is given on pages 7 - 8. This approach has been successfully used to implicate new genes in severe early-onset immune pathology and to elucidate disease pathogenesis and pathways involved in the illness, but also to gain insight in the functioning of the human immune system overall. For example, compound heterozygous loss-of-function mutations in *IRF7* (interferon regulatory factor 7) were recently found in an otherwise healthy child that suffered from life-threatening primary influenza infection [20]. Mechanistic studies revealed reduced type I and III interferon (IFN) production by leukocytes, plasmacytoid dendritic cells (pDCs), dermal fibroblasts and induced pluripotent stem cell (iPSC)-derived pulmonary epithelial cells of the patient as well as increased influenza virus replication in the latter two cell types. Together with the clinical phenotype these findings suggest that IRF7-dependent amplification of type I and III IFNs is required for protection against primary infection by influenza virus in humans [20].

Criteria for establishing a causal relationship between a candidate genotype and the clinical phenotype of a single patient.

1. **The candidate genotype must be monogenic, may not occur in healthy individuals and must have a frequency less than or equal to the frequency of the phenotype.**
    a. The clinical phenotype must be rare and distinctive and the candidate genotype must be monogenic.
    b. Family studies must demonstrate that the candidate genotype of the patient is not shared by other family members (complete clinical penetrance, Mendelian mode of inheritance).
    c. Population studies, including but not restricted to the same ethnic group, must indicate that the candidate genotype does not occur in healthy individuals. The frequencies of the candidate variants and genotype are not higher than that predicted by the frequency of the clinical phenotype.
2. **The genetic variant(s) must impair, destroy, or alter the function of the gene product.**
    a. Functional prediction algorithms are helpful but not conclusive. A variation that is not conservative and that occurs in a region or at a residue of the encoded protein that is highly conserved in evolution provides support for the hypothesis that the amino acid is functionally important.
    b. Studies should document whether the variant changes the amount or molecular weight of the gene transcript and of the encoded protein. Ideally, this should be done in control primary cells or iPSC-derived cell lines, and not only in control immortalized cell lines.
    c. The variants must be loss- or gain-of-function for at least one biological activity. For variants that result in an amino acid substitution, insertion or deletion, *in vitro* studies should document a functional change that reveals the mechanism by which the variant causes disease, e.g. the protein may be unstable, it may not bind essential cofactors, or it may not localize appropriately.
3. **The causal relationship between the candidate genotype and the clinical phenotype must be established via a relevant cellular or animal phenotype.**

a. The candidate gene should be known or shown to be normally expressed in cell types relevant to the disease process, e.g. cells affected by the disease process, cells which produce factors needed by the affected cells or progenitors of the cell lineage affected by the disease. Some genes are broadly expressed but have a narrow clinical phenotype.

b. For disorders that affect the function of a cell (present in the patient), experimental studies *in vitro* must indicate that there is a cellular phenotype explained by the candidate genotype, ideally in primary cells. This cellular phenotype should reasonably account for the clinical phenotype because the cell type is known to be involved in the disease process and the clinical phenotype is consistent with it.

    i. The cellular phenotype should be reproduced in independent cell lines: Overexpressing the mutant allele in a different system should reproduce the cellular phenotype observed in patient primary cells. For example, loss-of-function mutations due to loss-of-expression must be confirmed by overexpressing the mutant allele(s) and confirming absent expression, negative dominance must be established by co-transfecting the mutant and wild-type alleles into cells deficient for the gene product. As overexpression can alter the function of a gene, alternatively or additionally knockdown or knockout of the wild-type gene, or introduction of knock-in mutations in control cells, should reproduce the cellular phenotype.

    ii. The cellular phenotype must be rescued by a wild-type allele for loss-of-function mutations or by knockdown, knockout, or correction of the mutant allele for gain-of-function or dominant-negative mutations.

c. For disorders that affect the development of a cell (lacking in the patient), an animal model must indicate *in vivo* that there are causally related phenotypes that mimic the patient's phenotypes (molecular, cellular, and clinical) and that are explained by the candidate genotype. A biological phenotype underlying the patient's clinical phenotype must be replicated in the mutant animal.

Adapted from "Guidelines for genetic studies in single patients: lessons from primary Immunodeficiencies" by Casanova, J.L., *et al. J Exp Med*, 2014. **211**(11): p. 2137-49.

In another example, a homozygous missense mutation in *PRKCD* (protein kinase C, delta), encoding protein kinase δ (PKCδ), was identified in 3 siblings who presented with juvenile-onset systemic lupus erythematosus (SLE) [21]. Mechanistic studies demonstrated that reduced activity of PKCδ led to reduced apoptosis of (autoreactive) B cells and increased B cell proliferation with increased numbers of immature B cells. In PKCδ deficient mice, which exhibit a SLE phenotype, B cell expansion and a developmental shift toward immature and naïve B cells was observed as well. These findings indicate that PKCδ is crucial in regulating B cell tolerance and preventing self-reactivity in humans, and that PKCδ deficiency represents a novel genetic defect of apoptosis leading to SLE [21].

3. Overview of the clinical phenotypes under study

3.1 Severe early-onset systemic lupus erythematosus, IgA-deficiency and mild lower limb spasticity without neuroradiological manifestations

3.1.1 Systemic lupus erythematosus

SLE is a prototypic systemic multi-organ autoimmune disease that is characterized by the production of autoantibodies against nucleic acids and related nuclear proteins, immune complex deposition and systemic vasculitis [22, 23]. Organ systems typically affected include skin/mucosae (rash), joints (arthralgia/arthritis), kidneys (glomerulonephritis) and/or hematopoetic cells (anemia, leucopenia, thrombocytopenia) [7]. Upregulation of IFN responsive genes in patients' peripheral blood cells, the IFN signature, is associated with more severe disease involving the kidneys, hematopoetic cells and/or the central nervous system (encephalopathy, stroke) [22, 24]. Up to 40% of SLE patients can develop an antiphospholipid syndrome, when anti-phospholipid autoantibodies such as anticardiolipine antibodies or lupus anticoagulans interfere with coagulation leading to increased clot formation or thrombosis [25]. Childhood-onset SLE accounts for 5-10% of all cases, with a incidence of about 2.2/100,000 in children aged 3-18 years [7]. In the majority of paediatric SLE patients an IFN signature is found [23, 24], and the development of SLE in childhood and adolescence is typically associated with a more fulminant disease onset and course, more associated damage over time and a relatively high male-to-

female sex ratio [7]. Childhood-onset disease is associated with an increased mortality risk due to infections, cardiovascular, pulmonary or renal disease; and without treatment the 5-year mortality rate reaches 95% [26, 27].

SLE is assumed to arise through a complex interplay of environmental and genetic factors. Considering the increased disease severity and the shorter exposure time to environmental triggers, genetic factors are thought to be relatively more important in the pathogenesis of childhood-onset SLE [28]. Patients with monogenic causes of SLE often have severe early-onset disease and are thought to comprise ~1% of the adult SLE cohort [29]. Known monogenic defects associated with SLE include mutations in *DNASE1L3 (*deoxyribonuclease 1-like 3), *DNASE1, PRKCD, TREX1 (*three prime repair exonuclease 1), *TRAP (*triiodothyronine receptor auxiliary protein), *SAMHD1 (*SAM domain and HD domain-containing protein 1) and the complement deficiencies [28, 29]. Through the identification of these genes several mechanisms that contribute to the development of SLE have been identified. Defects in apoptosis and clearing of nuclear debris leads to production of double-stranded DNA (dsDNA) autoantibodies and autoimmunity. For example, recessively inherited loss-of-function mutations in *TREX1,* the main 3'-5' DNA exonuclease responsible for degrading DNA during granzyme A-mediated apoptosis, lead to accumulation of endogenous DNA and subsequent IFN-α production [30]. TREX1 deficiency is associated with SLE and familial chilblain lupus, but also with Aicardi-Goutières syndrome (AGS), a rare neuroimmunologic disorder associated with elevated levels of type I IFN and characterized by severe early-onset leucoencephalopathy, brain atrophy and intracranial calcifications leading to profound intellectual disability, spasticity and dystonia [31, 32]. Other mechanisms include defects in the clearing of apoptotic cell bodies and of circulating immune complexes as is seen in the complement deficiencies [33], or loss in B cell tolerance as is seen in PKCδ deficiency [21]. Additionally, several genetic variants associated with SLE susceptibility have been identified through genome-wide association studies (GWAS). Functional studies into the biological effects of these variants have implicated several other potential mechanisms and pathways in the disease pathogenesis of SLE, such as signalling through nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and Toll-like receptors (TLRs),

adhesion and migration of monocytes and neutrophils, B cell function (activation, proliferation, differentiation) and T helper cell (Th) function (Th1, TH17) [29].

Conventional treatment of SLE consists of immunosuppressive/immunomodulatory drugs such as corticosteroids, hydroxychloroquine, mycophenolate mofetil, cyclophosphamide or azathioprine, depending on disease presentation and severity [34]. Autologous hematopoietic stem cell transplantation (HSCT) has been used in patients with severe SLE refractory to immunosuppression and treatment toxicity, however more than 50% of the patients experienced a relapse [35]. Increased insight into the pathogenesis of SLE has driven the development of biologic therapies that target B cells (e.g. anti-CD20 mAb) or inhibit costimulatory molecules (e.g. anti-cytotoxic T-lymphocyte-associated protein 4 (CTLA4) mAb) or type I IFNs (e.g. anti-IFN-α/β receptor (IFNAR) mAb, anifrolumab) [34]. Clinical trials on the use of oral Janus kinase (JAK) inhibitors in SLE, to block the IFN signalling cascade downstream of IFNAR, are ongoing [36].

3.1.2 Selective IgA deficiency and autoimmunity

Immunoglobuline A (IgA), the most amply produced antibody isotype, serves as a first line of defence against inhaled and ingested pathogens at the mucosal surfaces of the respiratory, gastrointestinal, and genitourinary tracts [37]. Selective IgA deficiency (sIgAD), defined as IgA < 7 mg/dl with normal serum IgG/IgM and normal IgG antibody response to vaccination, is considered the most common immunoglobulin deficiency. The prevalence of sIgAD varies greatly among populations, from 1/142 in Arabian Peninsula to 1/18,500 in Japan [37]. The clinical presentation of sIgAD ranges from asymptomatic to recurrent sinopulmonary and gastrointestinal infections. Atopy and high IgE levels are also often found in sIgAD patients [38], but a striking finding is the presence of autoantibodies in about 40% of sIgAD patients, with or without clinical manifestations of autoimmune diseases such as autoimmune hypothyroidism, celiac disease, systemic lupus erythematosus or diabetes mellitus [37]. Many hypotheses have been put forward to explain the association between sIgAD and autoimmunity. Increased exposure to environmental antigens that could possibly cross-react with self-antigens could lead to an enhanced immune response and

development of autoimmunity, or defective antigen clearance could result in immune complex deposition in many organs leading to inflammation [37]. It has also been demonstrated that IgA has anti-inflammatory functions. Serum IgA is normally bound to its receptor on myeloid cells. While an activating response is obtained when IgA binds its antigen, binding of IgA alone to its receptor induces a strong anti-inflammatory action towards responses induced by other cell-surface receptors on phagocytes [37]. This dual function depending on binding of the receptor to uncomplexed or antigen-complexed IgA, may contribute to the balance between inflammatory and anti-inflammatory processes. Monogenic defects leading to sIgAD have not been identified yet, however, genetic variants associated with increased susceptibility to both sIgAD and autoimmunity have been identified through GWAS, the most important ones being major histocompatibility complex (MHC) haplotype 8.1 and polymorphisms in *CLEC16A* (C-type lectin domain family 16) and *IFIH1* (interferon induced with helicase C domain 1) [39].

3.1.3 Spastic paraparesis

Spastic paraparesis is a heterogeneous group of neurological disorders characterized by lower limb spasticity and weakness. This condition can be the result of intrauterine infections or neonatal asphyxia, and neuroimaging often shows abnormalities such as calcifications or periventricular leucomalacia [40]. Although they are much less prevalent than cerebral palsy, hereditary disease forms exist in which patients develop a progressive loss of corticospinal motor tract function [41]. In these neurodegenerative disorders neuroimaging is normal, symptoms occur later in life and they usually progress slowly and steadily. When symptoms begin in very early childhood, they may be non-progressive and resemble spastic diplegic cerebral palsy. The prevalence of hereditary spastic paraparesis (HSP) is estimated to be 1.2 - 9.6/100,000. More than 50 genetic HSP types have been described, and the associated genes are involved in neuronal axon transport, endoplasmic reticulum morphology, mitochondrial function, myelin formation, protein folding and endoplasmic reticulum-stress response, corticospinal tract and other neurodevelopment, fatty acid and phospholipid metabolism and endosome membrane trafficking and vesicle formation, as described in detail in the review by Fink *et al.* [41]. Recently, mutations in *ADAR1*,

IFIH1, and *RNASEH2B*, genes known to be involved in AGS, have been found in patients with HSP as well [42]. Neuroimaging was normal or showed nonspecific dysmyelination in one patient, and the patients with mutations in *ADAR1* and *IFIH1* showed a robust IFN signature in peripheral blood. This association between type I IFN and neurological phenotypes such as AGS or HSP suggests that type I IFN may act as a neurotoxin during prolonged exposure [42].

3.2 Castleman's disease

Castleman's disease is a rare lymphoproliferative disorder characterized by polyclonal B cell proliferation. It can be divided into unicentric Castleman's disease (UCD), when a single lymphoid region is involved, and multicentric Castleman's disease (MCD) in which multiple lymphoid regions are involved [43]. In UCD symptoms are mostly local due to enlargement of one or more lymph nodes and removal of the lymph node is often curative [44]. MCD on the other hand, is often associated with systemic symptoms [43] and systemic treatment is necessary [44]. Castleman's disease is characterized by hypersecretion of IL-6 without any significant production of other cytokines [45]. The overproduction of IL-6 is assumed to be responsible for systemic symptoms such as fever, polyclonal B-cell expansion and lymphadenopathy/hepatosplenomegaly, hypergammaglobulinemia and increased levels of acute-phase reagents [45, 46]. Before the development of biologicals, therapy consisted of corticosteroids, combination chemotherapy and/or lenalidomide/thalidomide [44]. Treatment with anti-CD20 mAb (rituximab) has been effective in Castleman's disease, especially in patients with pre-existing autoimmune disorders [47]. However, treatment with anti-IL-6 receptor (IL-6R) mAb (tocilizumab) has resulted in remarkable clinical improvement, consistent with the major role of IL-6 in disease pathogenesis [48, 49].

Castleman's disease is an orphan disease. Prevalence is estimated to be less than 1/100,000 [50]. The incidence of UCD is highest in the third and fourth decade of life, for MCD the peak of incidence lies in the fourth and fifth decade of life [44, 50]. Castleman's disease is even rarer in children, with only about 100 paediatric cases published, and the disease presentation in children is usually that of UCD [43, 50].

Infections with human herpes virus (HHV)-8 are responsible for approximately 50% of MCD cases [51], mostly in human immunodeficiency virus (HIV)-positive but also in HIV-negative patients, by driving hypercytokinemia secondary to the expression of the virus-encoded cytokine, viral IL-6 [52]. Therefore, MCD is often classified as HHV-8-positive MCD and HHV-8-negative or idiopathic MCD (iMCD). The genetic cause of iMCD remains to be elucidated. Functional polymorphisms in the human IL-6R leading to increased expression of soluble IL-6R have been found with increased frequency in patients with iMCD compared to healthy individuals [53]. This finding further illustrates the importance of the IL-6 pathway in disease pathogenesis. iMCD can be associated with pre-existing autoimmune disorders such as rheumatoid arthritis, Sjögren's syndrome and SLE [54]. Hypercytokinemia in iMCD is thought to be the consequence of either systemic inflammatory disease mechanisms via autoantibodies or inflammatory gene mutations, of paraneoplastic syndrome mechanisms via ectopic cytokine secretion, and/or a of non-HHV-8 virus [55].

3.3 Combined immunodeficiency, lymphoproliferation, autoimmunity and vasculopathy of the central nervous system

3.3.1 Combined immunodeficiency with autoimmunity and lymphoproliferation

Combined immunodeficiencies (CIDs) comprise of a heterogeneous group of disorders of cellular immunity that are caused by either a deficient number or deficient function of T lymphocytes [56]. In contrast with severe combined immunodeficiency (SCID), in which profound T cell lymphocytopenia is present, CID patients have a significant number of (potentially defective) autologous circulating T cells, although they frequently lack naïve T cells [57]. More than 70 different genetic defects responsible for T cell dysfunction have been identified [56]. These genetic defects can lead to impairment of different types of T cell activity, such as T cell development, antigen presentation, activation, costimulation, proliferation/metabolic adaptation, quiescence, survival/migration, memory generation/maintenance, (general or selective) effector functions, senescence and regulatory functions. CIDs are accompanied by a humoral defect as well, either due to intrinsic B cell defects or due to the lack of T cell help for antibody production [58]. The nature of the genetic defect

might explain differences in clinical presentation of CIDs, however, all of these disorders are associated with susceptibility to both bacterial and (severe) viral infections, and in some cases fungal and mycobacterial infections as well. Given the broad definition of CID, it is difficult to estimate the prevalence of this group of disorders. Newborn screening in the United States of America identified SCID in 1 in 58000 births, however prevalence of non-SCID T cell lymphopenia varied from 1/2100 to 1/14000 depending on the state although the majority of these cases was not associated with T cell dysfunction [59]. The prevalence of common variable immunodeficiencies (CVID), an equally heterogeneous group of disorders characterized by decreased serum immunoglobulins, poor specific antibody response upon vaccination and recurrent bacterial infections, is estimated to range from 1/25000 to 1/50000 [60].

CID is often associated with autoimmunity and lymphoproliferation, since self-tolerance relies on an intact immune system to suppress autoreactivity and the present T cells can expand and attack normal tissue [56]. This seems evident when the genetic defect has an effect on regulatory functions of T cells. For example loss-of-function mutations in *FOXP3* (forkhead box P3), the master transcription factor for the development and function of regulatory T cells (Tregs), were identified in patients with the 'immune dysregulation, polyendocrinopathy, enteropathy, X-linked' (IPEX) condition [61]. In the most severe cases patients present with very severe and early-onset gut disease (probably initiated by excessive inflammation triggered by the gut microbiota), food allergies and in a later stage type I diabetes. However, defects in other T cell functions can lead to autoimmunity and lymphoproliferation as well. For example genetic defects leading to impaired signalling downstream of the T cell receptor (TCR) after antigen recognition almost always present with severe impairment of T cell immunity but also with autoimmune features. This can be explained by changes in TCR signalling strength that can modify the threshold for negative selection of self-reactive T cells in the thymus and/or cause impaired production of Tregs.

Depending on the nature and severity of the immune defect, different forms of treatment are indicated for CID. In general, patients receive intravenous

immunoglobuline (IVIG) replacement therapy in order to maintain protective IgG serum levels [58] and most patients also receive infectious prophylaxis [58]. When autoimmune/autoinflammatory manifestations are present, addition of immunosuppressive treatment is necessary [60]. A wide range of immunosuppressive regimens are available but often fail in controlling the disease. Insight in the pathogenic mechanisms involved can help in choosing a correct therapeutic approach. For example, in autoimmune enteropathy the disease process seems to be mostly driven by T cells producing Th1 cytokines such as IL-12 and IFN-γ [62]. Therefore, T cell-targeted therapies such as calcineurin inhibitors are often used once steroid treatment has failed [60]. For severe T cell deficiencies or in case of treatment failure, allogeneic HSCT can be curative and lead to a life-long restoration of a functionally normal adaptive immune system [63]. Success of the HSCT in CID depends on the availability of a human leukocyte antigen (HLA) compatible healthy donor and the effectiveness in controlling pre-existing infections and immune dysregulation [63]. Due to the risks associated with (non-HLA-matched) allogeneic HSCT, efforts are being made to introduce gene therapy, e.g. correction of the genetic defect in the patient's own hematopoietic stem cell, in the clinical setting [64].

3.3.2 Early-onset stroke and vasculopathy/vasculitis of the central nervous system

Although stroke is often considered to be associated with old age, it can occur in children as well. The incidence of stroke in children aged 0-19 years is estimated at 5 out of 100,000 [65]. The risk of childhood stroke is greatest in the first year of life, and peaks during the perinatal period [65]. Risk factors for stroke in children include congenital or acquired (e.g. infectious) heart disease, anatomical malformations of central nervous system (CNS) blood vessels, CNS infections, head or neck trauma, haematological disorders such as sickle cell anaemia or haemophilia, or autoimmune/autoinflammatory disorders. In autoimmune/autoinflammatory disease, the cause of the stroke can be blood vessel-related, i.e. stroke as a consequence of CNS vasculitis or vasculopathy, or due to the development of a coagulation disorder (e.g. antiphospholipid syndrome in SLE) [56].

Primary systemic vasculitis, with our without CNS involvement, is a heterogeneous group of disorders characterized by blood vessel inflammation [66]. Most of the primary vasculitis syndromes are rare in childhood, but are associated with significant morbidity and mortality [66]. CNS vasculitis can occur in the context of systemic vasculitis with a CNS component, but isolated disease only affecting the CNS exists as well, e.g. childhood primary angiitis of the central nervous system (cPACNS). Several genetic defects that are associated with CNS vasculitis or vasculopathy have been identified. Intracerebral arteriopathy and early-onset stroke is commonly found in patients with recessively inherited loss-of-function mutations in *SAMHD1*, one of the disease-causing genes in AGS [67]. Large artery disease is not observed in patients with mutations in other AGS-associated genes, perhaps indicating a particular role for *SAMHD1* in blood vessel integrity. Recently, dominantly inherited loss-of-function mutations in *TNFAIP3* (tumor necrosis factor, alpha-induced protein 3) leading to A20 haploinsufficiency were identified in patients with Behçet-like disease, with 1 patient suffering from CNS vasculitis as well [68].

Zhou *et al.* identified recessively inherited loss-of-function mutations in *CECR1* (cat eye syndrome chromosome region, candidate 1), encoding adenosine deaminase 2 (ADA2), in patients with systemic inflammation, systemic vasculopathy in the form of early-onset recurrent stroke, livedoid rash or vasculitis, hepatosplenomegaly, hypogammaglobulinemia and lymphopenia [69]. Simultaneously, Navon Elkan *et al.* identified recessively inherited loss-of-function mutations in *CECR1* in patients with systemic polyarteritis nodosa, a necrotizing vasculitis [70]. Patients presented with systemic inflammation, livedoid rash, peripheral neuropathy and systemic vasculitis/vasculopathy, mostly visceral involvement but several patients presented with CNS vasculopathy and early-onset stroke as well. All ADA2-deficient patients had reduced levels of ADA2 as well as a reduction in ADA2-specific enzyme activity in the blood. Cytokine profiles were normal. Brain biopsies revealed vasculopathy changes characterized by compromised endothelial integrity, endothelial cellular activation and inflammation. *In vitro* experiments demonstrated that patient monocytes preferentially differentiated into M1 macrophages and induced damage in cocultured endothelial-cell layers. Therefore it was hypothesized that possible disease

mechanisms in ADA2 deficiency are proinflammatory macrophage polarization and disturbed endothelial integrity.

3.4 Severe viral illness

Although many forms of PID are associated with severe, persistent, recurrent, or refractory viral illness, isolated susceptibility to viral infections is considered to be rarer [71]. While in the past susceptibility to severe viral diseases was thought to be the consequence of deficient lymphocyte or innate immune cell responses, especially $CD8^+$ T cell or natural killer (NK) cell responses [56, 71], several defects in innate pathogen recognition receptors [72] and signalling molecules [20] have been identified that can lead to either pathogen-specific susceptibility or predisposition to severe disease associated with multiple viruses [71]. It is difficult to estimate the prevalence of disorders due to isolated susceptibility to viral infections. Life-threatening disease is often wrongfully contributed to bacterial disease, and viral infection can be more difficult to document as pathogen-specific tests by polymerase chain reaction (PCR) are mostly used for diagnosis in current practice. For specific disease entities such as herpes simplex encephalitis (HSE), a well-defined and well-documented disease caused by a pathogen-specific susceptibility to herpes simplex virus (HSV)-1, an estimated prevalence of about 1/10,000 has been established and mutations in six genes of the TLR3 signalling pathway have been identified in children with HSE [73].

Many of the genetic defects in innate immunity identified in patients with isolated viral susceptibility, are associated with a failure in the induction of, signalling of or response to type I IFN. For example, both defects in the TLR3 signalling pathway in HSE and genetic defects in IRF7 in a patient suffering from life-threatening influenza infections lead to a failure to induce type I IFN [20, 73]. Increased susceptibility to measles can be associated with either signal transducer and activator of transcription 2 (STAT2) deficiency or IFNAR deficiency, both leading to a failure to respond to this group of cytokines [74, 75]. Additionally, susceptibility to viral infection has been observed in mice with genetic defects in the IFN signalling pathway, for example deficiency of Ifnar1 [76] or Stat1 [77]. Furthermore, most viruses have developed strategies to evade the IFN response and lose pathogenicity when their IFN antagonists

are knocked out [78]. All these findings demonstrate the importance of the type I IFN response in innate antiviral defence.

Prevention forms an important part of the management of PID patients with or without isolated increased susceptibility to viral illness. Non-live vaccines can significantly reduce the number of viral infections in PID patients, for example in the IRF7-deficient patients annual influenza vaccination as sole secondary prevention was sufficient to prevent further disease [20]. The administration of live-attenuated vaccines is contraindicated in PID patients, especially in those patients with an increased susceptibility to these pathogens [79]. Vaccination of the family and surroundings as well as sufficient population vaccination coverage is necessary to provide herd immunity to those patients. Patients can receive prophylactic IVIG treatment, in the hope of preventing severe and recurrent viral illness [80]. Additionally, environmental exposure to viral pathogens should be avoided when possible. However, once a severe viral illness has developed, treatment is challenging. Several antiviral drugs are available that do not destroy the pathogen but intervene in viral replication and can therefore assist in controlling the disease, for example acyclovir, a nucleoside-analog (9-(2-hydroxyethoxymethyl) guanine) that specifically inhibits replication in herpes viridae [81]. For specific pathogens monoclonal antibodies have been developed that help clear the infection, for example palivizumab, a monoclonal antibody against respiratory syncytial virus (RSV) [82]. Otherwise, treatment remains limited to intensive supportive care.

OBJECTIVES

The main objective of this project is to identify novel genes capable of causing monogenic defects in the human immune system by studying paediatric patients with severe immunological disorders. The project received approval by the ethical committee of the University Hospital of Leuven, Belgium (study reference number: S52653). By collecting both genetic and immunological data from patients with distinct clinical manifestations belonging to the whole spectrum of immune dysregulated conditions (both hypo & hyper immune states) but without a genetic diagnosis, strong associations between clinical phenotype, immune phenotype and genotype are searched for.

To clarify the contribution of novel genes in severe immunological disorders, the following sub-objectives are formulated:

1. to identify candidate genetic variants responsible for severe early-onset immunological disorders in humans
2. to investigate alterations in the peripheral immune system of patients with these disorders in order to gain insight in pathways involved in disease pathogenesis
3. to verify the role of a candidate genetic variant in disease pathogenesis and prove it is causal

METHODOLOGY

1. Genetic analysis: identification of a candidate genetic variant

1.1 Sequencing techniques

The development of 'next generation sequencing' (NGS) techniques such as whole-exome sequencing (WES) and whole-genome sequencing (WGS) have revolutionized genetic studies by allowing detailed exome- or genome-wide screening for disease-causing variants [83-85]. In WES the protein-coding regions of the genome are sequenced [83]. Although these regions make up approximately 1% of the human genome, it is estimated that 85% of the disease-causing mutations are located in coding regions of the genome [86], rendering WES a distinct option in the search for disease-causing variants. Disadvantages of WES compared to WGS include incomplete coverage of the target region, difficulties in detecting copy number variants (CNV) as well as large insertions and deletions, and of course the inability to detect variants in promoter sequences, intronic regulatory sequences or other regulatory sequences such as long non-coding RNAs or microRNAs. The major advantages of WES over WGS are the lower cost, the shorter sequencing time and the smaller, more manageable data set for faster and easier analysis [87].

Based on the arguments stated above as well on the availability of the technique in our research institution, WES was chosen to screen the entire human exome and the adjacent intronic regions containing splice sites for possible disease-causing genetic variants. Given the severe clinical phenotypes in our patient cohort, we hypothesized that there is a high chance that the causative mutations are located in the coding regions of the genome and that this strategy would therefore be successful. It is important to mention however, that due to its rapidly decreasing costs and the development of faster sequencing methods, WGS is becoming an increasingly attractive alternative for WES [88]. Although it remains difficult to interpret variants in intronic or intergenic regions, WGS is a superior technique for the detection of CNVs and it provides increased coverage of the target region in comparison with WES. Furthermore, recent studies have demonstrated that WGS is more efficient in calling high-quality coding single nucleotide variants (SNV) as well [88], suggesting that WGS

might be a more powerful technique than WES for detecting potential disease-causing mutations in the targeted exome.

1.2 Selection of a candidate genetic variant

The technical aspects of target enrichment and massive parallel sequencing in WES have been described in detail before [83]. Briefly, genomic DNA is fragmented and an

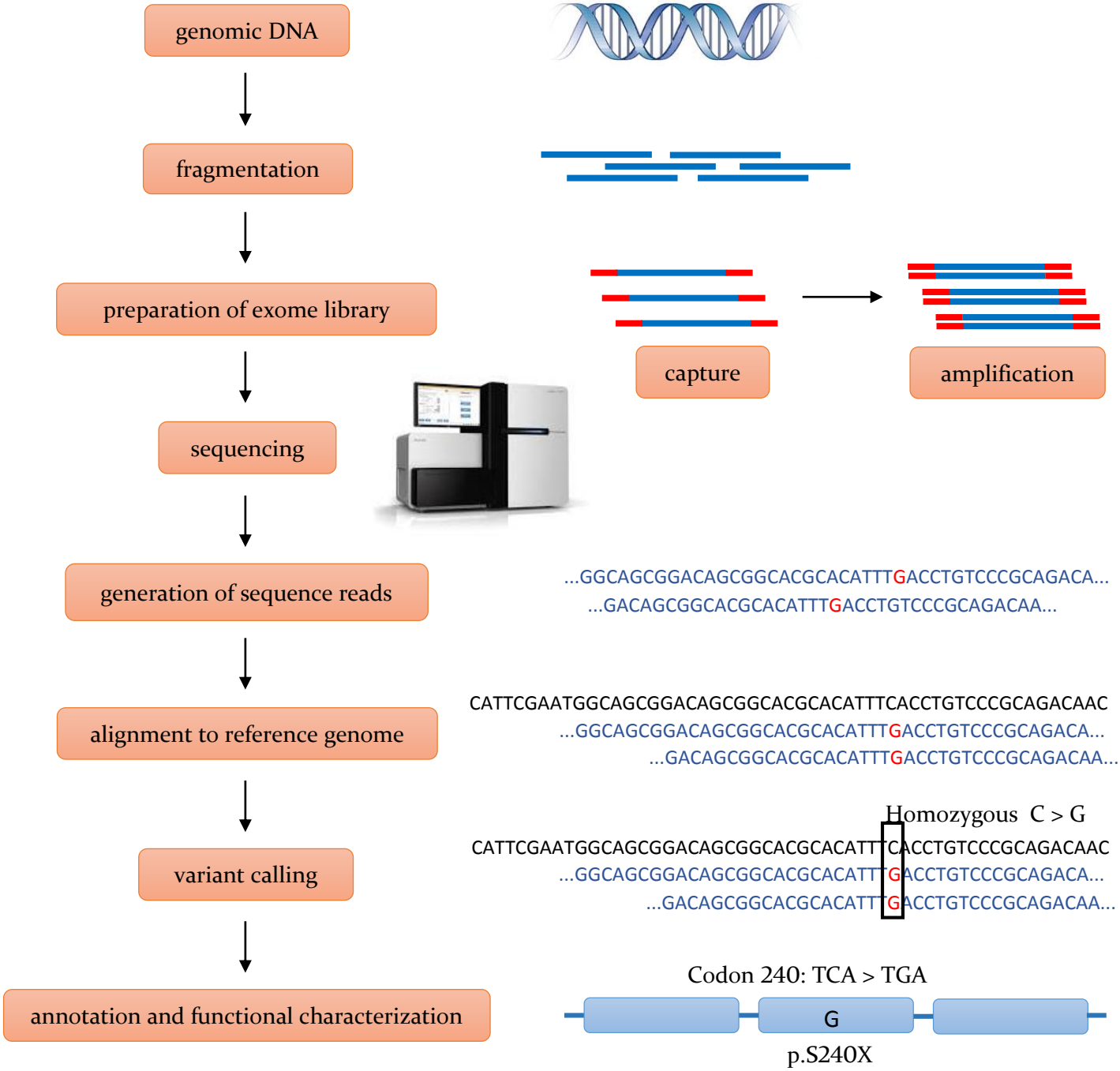

Figure 1: **Steps for generating whole-exome sequencing data.**

exome sequence library is prepared by capturing the target region (exome) and amplifying it by PCR. After sequencing of the sample, the fragment reads are aligned to the reference genome, single nucleotide variants and insertions/deletions are identified and the variants are annotated. Figure 1 shows a schematic overview of the basic steps for generating WES data. A detailed description of the technical aspects of the technique is mentioned in the methods section of each publication in this manuscript. The selection of candidate genetic variants is an important part of this work and therefore the principles and current practices for achieving this are discussed in more detail. An overview of the necessary steps for selecting a candidate genetic variant can be found in Figure 2.

1.2.1 Selection of rare variants

WES can generate up to 20,000 genetic variants per individual compared to the reference genome [89]. When selecting candidate disease-causing genetic variants in patients suffering from rare diseases, it is important to focus on rare genotypes as well. Therefore, genotype and allele frequencies are important factors, and filtering out common polymorphisms is the first step in narrowing down the number of genetic variants in an individual. This can be achieved by searching for the variant in multiple online databases such as 1000Genomes [90], NHLBI ESP6500 [91], HapMap [92], dbSNP [93] or ExAc [94]. These public databases contain the frequencies of genetic variations of a large number of individuals of various ethnicities that were obtained using NGS techniques. It is important to mention that public databases can contain disease-causing variants as well. Therefore, excluding all variants that are found in these databases might be too strict, especially in the case of rare disorders with a suspected recessive inheritance which can be caused by a rare variant that can be found in control heterozygotes as well. Genotype frequency is more relevant than allele frequency, and depending on the suspected mode of inheritance and the expected frequency of the phenotype, variants with a low frequency in public databases (e.g. control allele frequency < 0,5% or 1%) should be included in the analysis as well. Furthermore, an in-house database that contains data from ethnically matched DNA samples analysed using the same technology is a powerful tool for excluding common polymorphisms specific for the examined population and has been used in this study.

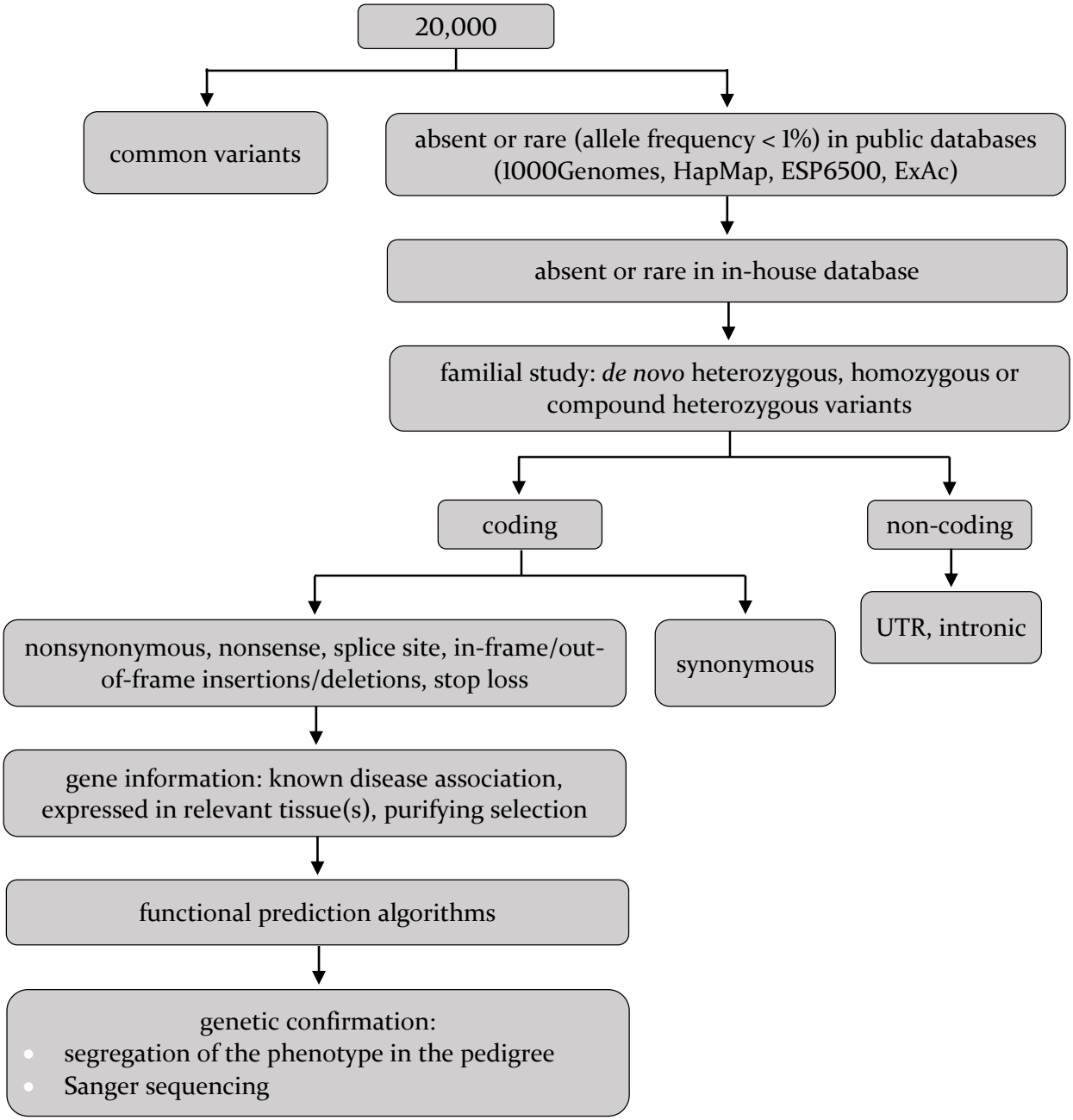

Figure 2: **Filtering steps of exomic variants for the identification of candidate genetic variants Mendelian disease mutations or rare variants with large effects.**

1.2.2 Family studies

Even after filtering out common polymorphisms, 500-1000 rare variants can still be found in one individual [89]. In the context of a rare, possibly Mendelian disorder in a single patient or family, WES is preferentially performed on a trio consisting of the patient and both parents. Based on the observed pattern of inheritance in the family, analysis of the exome data of all three individuals is performed to identify rare *de novo* heterozygous or rare inherited homozygous or compound heterozygous genetic variants. Previous familial studies have shown that one trio contains on average 12 rare genotypes: one *de novo* genotype (absent in control population), one hemizygous genotype (control allele frequency < 1%, never observed in male controls and never homozygous in female controls), three homozygous and seven compound heterozygous genotypes (control allele frequencies < 1%) [95]. Even when using less stringent criteria, the familial study approach will lead to a manageable list of candidate variants. When examining extremely consanguineous families with a large number of homozygous variants, additional WES of healthy siblings offers the most added information, since this data can be used to exclude homozygous variants that are present in the healthy siblings and are therefore most likely not disease-causing.

1.2.3 Prioritizing candidate genetic variants

Next, the remaining candidate genetic variants must be prioritized and the most plausible one selected for further mechanistic studies. A variant can be selected or filtered out based on available information of the mutated gene, and/or based on the predicted impact of the variant on gene function.

Several arguments based on available gene information can be made for selecting a candidate disease-causing gene:

1. The protein encoded by the gene belongs to a pathway already implicated in patients with a similar phenotype. Databases and software such as 'The human gene connectome server' (HGCS) that allow researchers to prioritize a list of genes by their biological proximity to defined core genes (i.e. genes that are known to be associated with the phenotype) and to predict novel gene pathways, are freely available [96].

2. Gene transcripts are expressed in tissue affected by the phenotype or in tissues that could influence or contribute to the phenotype. Public databases containing information on gene expression in a wide array of human or murine cell lines, cell types, tissues and organs are available and easy accessible [97, 98].
3. Genes containing multiple deleterious mutations in the general population are unlikely to be responsible for a rare, severe phenotype with complete penetrance. A database and software for the calculation of the nonsynonymous mutational load in each protein-coding gene in the general population (i.e. the gene damage index) are freely available [99]. In contrast, variants in genes under tight purifying selection are more likely causative of rare, severe early-onset diseases. Statistical methods for detecting the different types of selection have been developed and for most human genes the extent of purifying selection is now known [100].

The predicted impact of the variant on gene function can also be used to (further) prioritize candidate genetic variants. Nonsense mutations, in-frame and out-of-frame insertions and deletions, and mutations of the stop codon (stop loss) are likely to be deleterious. The impact of missense mutations can vary greatly and their pathogenicity can be predicted using functional prediction algorithms such as SIFT [101] and Polyphen-2 [102] that are based on a combination of biochemical and evolutionary data. The CADD algorithm scores the deleteriousness of both SNVs and insertions/deletions by weighing and integrating multiple annotations/scores of the variants [103]. Splice site variants that disrupt the consensus dinucleotide splice sites (+/-1 and +/-2) are also likely to be pathogenic. For variants located further away from the intron/exon boundary prediction algorithms exist as well, e.g. Human splicing finder 3.0 [104], NetGene2 [105] or NNSplice [106]. In contrast, UTR variants and intronic variants far beyond acceptor and donor sites at intron/exon boundaries are difficult to interpret in single patients, although they can interfere with splicing and other regulatory processes as well. Synonymous variants are usually considered far less likely to be pathogenic and are often filtered out, however, they can be disease-causing as well through several mechanisms:

1. **Alternative splicing**: A synonymous variant can lead to the creation of a new splice site. A mild form of cystic fibrosis caused by alternative splicing due to a synonymous c.2811G>T variant (p.Gly893Gly) in *CFTR* (cystic fibrosis transmembrane conductance regulator) has been described [107]. It seems imaginable that some synonymous variants might have some subtle effect on splicing without actually creating or destroying a splice site as well.
2. **Alterations in gene expression through altered interaction with microRNAs**: A synonymous c.313C>T variant (p.Leu105Leu) in *IRGM* (immunity-related GTPase M), encoding a regulator of autophagy, leads to an increased risk of developing Crohn's disease due to reduced binding with miR-196 [108]. Specifically, miR-196 binds well to the C allele, thus downregulating *IRGM*, but not to the T allele. The T allele therefore leads to higher disease risk because overexpression of IRGM reduces the cell's ability to autophagize invading bacteria.
3. **Altered secondary mRNA structure**: Synonymous variants can impact the efficiency of translation and thus the amount of protein produced. A synonymous c.474A>C variant (p.Val158Val) in *COMT* (catechol-O-methyltransferase), encoding a protein implied in catecholamine inactivation, leads to increased stability of the secondary mRNA structure and therefore reduced amounts of translated protein, reduced enzymatic activity and increased pain sensitivity in individuals carrying this variant [109]. By influencing the speed at which translation proceeds along the mRNA and since proteins begin to fold while they are still being translated, synonymous variants can lead to totally different protein folding as well.

Other mechanisms might exist through which synonymous variants can be harmful. In *Drosophila melanogaster* 22% of synonymous sites evolve under very strong selective constraint and are highly conserved [110]. Genes enriched in strongly constrained synonymous sites are often involved in key developmental pathways [110]. The observed purifying selection acting on synonymous sites is likely not limited to Drosophila, and an important role of synonymous variants in human genetic disease should be considered. However, due to the difficulty in interpreting synonymous

variants and the expert opinion that the majority of synonymous variants found in a genome are less likely to be responsible for severe, early-onset immune diseases, they were excluded from analysis in a first step.

1.2.4 Genetic confirmation of the candidate genetic variant(s)

Once a candidate genetic variant/genotype is identified, it is first confirmed by Sanger sequencing. False positive findings due to technical aspects of WES (e.g. incorrect nucleotide incorporation during target enrichment, assembly misalignment) are possible and therefore a variant has to be confirmed by use of an alternative sequencing technique [83]. In a next step, segregation of the variant/genotype with the phenotype in the pedigree is investigated. For single patient cases complete penetrance is a necessary requirement [14]. Discovery of the same genotype of a single patient in an unaffected individual of the family should be a reason for excluding the candidate genetic variant(s), until unrelated individuals with a similar phenotype and variants in the same gene are found.

2. Immunophenotyping: screening for alterations in the peripheral immune system

Advanced analysis of the peripheral immune system of patients and healthy individuals is performed, in order to identify alterations in the patient immune system compared to that of healthy age-matched individuals. The hypothesis is that the observed alterations in the peripheral immune system, i.e. the patient's immune phenotype, can provide insight in disease mechanisms and pathways involved in disease pathogenesis. For example, the observed shift towards immature B cells in PKCδ-deficient patients who suffer from SLE together with a similar observation in PKCδ-deficient mice demonstrates the importance of PKCδ for mature naïve B cell differentiation and for the regulation of B cell tolerance [21]. The obtained information on alterations in the patient immune system can also be useful in the selection of candidate genetic variants, when for example one gene out of a list of candidate genetic variants is expressed in an immune cell type that is (severely) affected in the patient(s).

However, careful interpretation of the observed alterations is necessary, since other factors, both genetic and environmental, can influence the function of the peripheral immune system and the appearance of an immune phenotype as well. Genetic regulation of the immune phenotype by relatively common genetic variants identified by GWAS has been demonstrated previously, when 23 independent variants at 13 loci where found to be associated with changes in the percentage of certain immune cell subsets [111]. For example, variants downstream of the *HLA-DRA* gene, previously identified as risk alleles for ulcerative colitis [112], systemic sclerosis [113], Parkinson´s disease [114], and Hodgkin´s lymphoma [115], are associated with decreased levels of memory $CD8^+$ T cells that do not express the costimulatory molecule CD28 ($CD45^+CD28^-CD8^+$ cells) [111]. Additionally, environmental stressors such as pathogens, toxins and/or medication can affect an individual's immune phenotype as well.

While gender typically has negligible effects on the immune phenotype, important age-related changes in proportions and function of different immune cell subsets have been demonstrated previously. The influence of old age, i.e. the decline in immune function in the elderly, has been studied extensively. For example, profound changes in T cell function and subsets such as a reduction in naive $CD8^+$ T cells are observed in elder individuals and are thought to be responsible for the reduced vaccination success in this population [116, 117]. Although age-related changes in the immune phenotype from infancy to young-adulthood have been investigated less, studies in different ethnic populations demonstrate similar findings: a decline in percentages of naïve $CD4^+$ and $CD8^+$ T cells and naïve B cells with age accompanied by an increase of memory $CD4^+$ and $CD8^+$ T cells and memory B cells indicating active development of the immune system, greater innate immune responses to TLR agonists in young children while regulatory responses (e.g. percentages of $FoxP3^+$ Tregs) decrease with age, an increase in T cell percentages and decrease in B cell percentages with age,... [118-120]. These findings underline the importance of using age-matched healthy individuals as controls when examining the peripheral immune system [121].

When examining the peripheral immune system, both cellular (e.g. lymphocytes, monocytes) and humoral (e.g. immunoglobulines, autoantibodies, cytokines) immune

components can be studied. The immunological profiling in this work falls along three basic categories: (1) analysis of blood plasma, (2) *ex vivo* leukocyte analysis and (3) *in vitro* analysis of stimulated leukocytes. By studying both cellular and humoral immune components of patients with severe early-onset immune pathology, both adaptive and innate immune system abnormalities are investigated in these disorders. Measurement of humoral immune components such as autoantibodies, antibody isotype levels and serum acute-phase reactants in patient plasma and/or serum is performed by the laboratory of the University hospital of Leuven. When necessary, cytokines can be measured in patient plasma and/or serum by Elisa, an assay that is still considered to be the gold standard for the detection and quantitative measurement of a specific substance in a liquid sample.

A human immunophenotyping platform using multicolour flow cytometry was previously developed in the Autoimmune Genetics Laboratory at KU Leuven, and is used to screen for immune cell alterations (i.e. changes in relative percentages of immune cell subsets) in the examined patients compared to healthy age-matched controls. Given the large variability and heterogeneity in the human immune system, even in a healthy population, accurate measurement of variations in the human immune system requires the use of standardized assays [122]. Figure 3 represent the workflow of the immunophenotyping platform. Flow cytometry is a powerful tool for analysing the function and the phenotype of immune cells, as it allows for the characterization of many subsets of cells in a complex mixture such as blood. Flow cytometry can be used to assess the expression of both cell-surface proteins and intracellular (phospho)proteins as well as other functional readouts [123, 124]. Through use of this technique relative percentages of subsets of peripheral blood mononuclear cells (PBMCs) are assessed, i.e. B and T lymphocytes, NK cells, NKT cells and dendritic cells (DCs). Within the B and T lymphocyte compartment, several developmental stages of B and T cells (e.g. naïve vs antigen-experienced) as well as Th subsets are quantified. Table 2 contains an overview of the immunological profiling with the markers used to define the different cell populations.

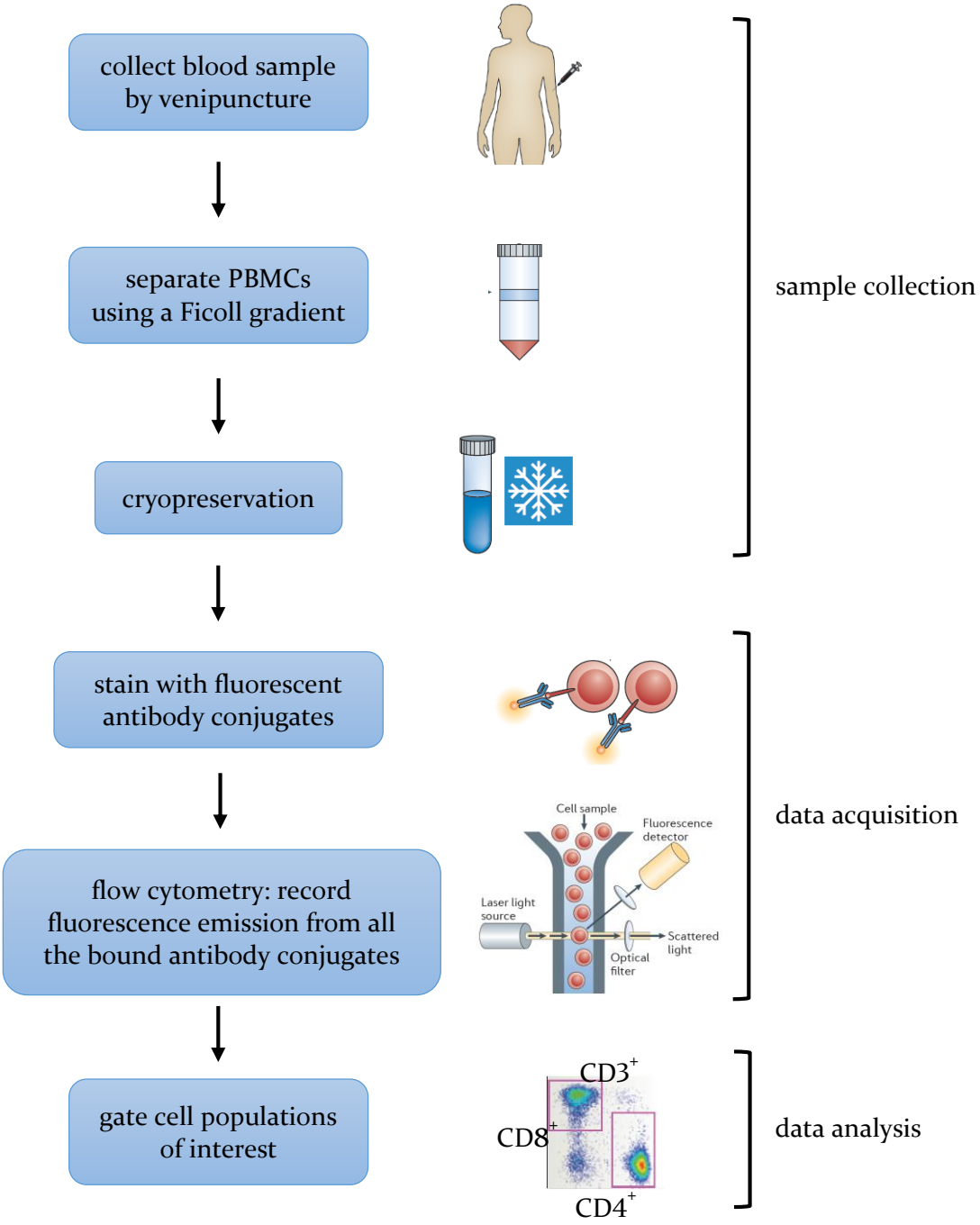

Figure 3: **Workflow of the immunophenotyping platform.** PBMCs, peripheral blood mononuclear cells. Adapted from "Standardizing immunophenotyping for the Human Immunology Project" by Maecker H.T., *et al. Nat Rev Immunol.* 2012 Feb 17;12(3):191-200.

Table 2: Advanced analysis of the peripheral immune system

Blood plasma analysis	
UZ Leuven laboratory	autoantibodies (ANA, ANCA, …) serum proteins (CRP, ferritin, …) antibody isotype levels
Cytokine profiles measured by Elisa	IL-6, IL-10, TNF-α, IFN-α, IFN-γ, IL-17A, …
Direct *ex vivo* analysis of PBMCs using flow cytometry	
Quantification of PBMC subsets	NK cells (CD4-CD8-CD3-CD19-CD14-CD56+) NKT cells (CD4-CD8-CD3+CD19-CD14-D56+) myeloid dendritic cells (CD19-CD3-CD14-CD56-CD11c+HLA-DR+) plasmacytoid dendritic cells (CD19-CD3-CD14-CD56-CD11c-CD123+HLA-DR+)
Quantification of T cell subsets	recent thymic immigrants (CD4+ or CD8+ CD45RA+CD31+) regulatory T cells (CD4+CD25hiIL7RAloFoxp3+) double negative T cells (CD4-CD8-CD3+CD56-) follicular T cells (CD4+CXCR5+CD45RO+) γδ T cells (CD3+γδ+) naive T cell (CD4+ or CD8+ CD45RA+) antigen-experienced T cells (CD4+ or CD8+ CD45RA-)
Quantification of B cell subsets	naive B cells (CD19+CD27-IgM+) IgM memory B cells (CD19+CD27+IgM+) switched memory B cells (CD19+CD27+IgM-) IgE+ B cells (CD19+IgE+) plasmablasts (CD19+CD24-CD38hi) transitional B cells (CD19+CD24hiCD38hi)

In vitro analysis of stimulated PBMCs	
TH1/TH2/THI7/Tfh quantification	5 hour PMA and ionomycin restimulation in Golgi-stop followed by intracellular cytokine staining for IFN-γ, IL-4, IL-2 and IL-I7

ANA, antinuclear antibodies; ANCA, antineutrophil cytoplasmic antibodies; CRP, C-reactive protein; IL, interleukin; TNF-α, tumor necrosis factor alpha; IFN-α, interferon-alpha; IFN-γ, interferon-gamma; PBMCs, peripheral blood mononuclear cells; NK cells, natural killer cells; NKT cells, natural killer T cells; PMA, phorbol myristate acetate.

3. Verification of the role of a candidate genetic variant in disease pathogenesis and proof of causality

When a candidate genetic variant or a list of candidate variants is available, functional tests are developed on a case by case basis in order to prove that a variant is disease-causing. For each examined case, specific tests are described in detail in the corresponding chapters. The underlying principles and steps necessary for addressing these scientific aspects are described here and are based on the paper 'guidelines for the genetic studies of single patients' by JL Casanova *et al*. 1) which states that, after identification of the candidate genetic variant(s), functional and mechanistic studies must (1) demonstrate that the genetic variant(s) impair, destroy, or alter the function of the gene product, and (2) establish a causal relationship between the candidate genotype and the clinical phenotype via a relevant cellular or animal phenotype [14].

3.1 The genetic variant(s) must impair, destroy or alter the function of the gene product

In a first step, the impact of the variant(s) on gene expression is tested. A possible limitation here could be that the gene is only expressed in difficultly obtainable cell types, or only during specific developmental stages. In the study of immunological diseases however, many of the relevant cell types are easy accessible by a simple blood sample. Changes in the amount or molecular weight of the gene transcript can be assessed. For protein-coding genes, protein expression can be examined on patient primary cells, or on recipient cells transfected with N- or C-terminal tagged protein if there is no antibody to the protein of interest available. Reduced or absent gene

expression is often the first experimental evidence that the variant(s) could be disease-causing.

In a second step, *in vitro* studies must demonstrate that the variants are loss- or gain-of-function for at least one biological activity. Loss of protein expression of course correlates with loss-of-function of the protein, however this can lead to (increased) activation of a pathway if the protein has an inhibitory function. For expressed proteins, *in vitro* studies should reveal a functional change and the corresponding mechanism by which the variant causes disease. Examples of such mechanisms could be failure of the protein to localize to certain cell compartments, failure to bind cofactors or target proteins, or aberrant constitutive activity of the protein. In general, functional studies are more difficult to conduct than expression studies because they require knowledge on the function of the examined gene and availability of specific tools. For example, examining chances in location or distribution of the protein within the patient cells can be done by immunofluorescence microscopy, however this requires a specific antibody.

Ideally, all biochemical studies of a candidate mutant allele should be performed in comparison with rare or common mutant alleles found in individuals without the phenotype under study that serve as negative controls.

3.2 The causal relationship between the candidate genotype and the clinical phenotype must be established via a relevant cellular or animal phenotype

A first step consists of the identification of an *in vitro* cellular phenotype for the patient: relevant, ideally primary patient cells should demonstrate a functional abnormality that is caused by the candidate genetic variant(s). This cellular phenotype should be able to explain the clinical phenotype of the patient, for example because the cell type is affected by or is known to be involved in the disease process. As mentioned earlier, a wide range of immune cell types are easily accessible by blood sample. Dermal fibroblasts are another primary cell type that is relatively easy to obtain by skin biopsy. The development of iPSCs-technology has enabled the study of other non-hematopoietic cell types that might be affected by disease as well, such as neurons [125], hepatocytes [126], cardiomyocytes [127], or respiratory epithelial cells [128]. In

immune pathology, this approach was used to demonstrate impaired intrinsic immunity and enhanced HSV-1 growth in patient-specific, iPSC-derived TLR3-deficient neurons and oligodendrocytes [129].

When such an *in vitro* cellular phenotype is available, establishing the causal relationship between genotype and phenotype can be done in various ways depending on the nature of the genotype:

1. *A rescue experiment that counteracts the effect of the genetic variant(s) must correct the cellular phenotype.* For loss-of-function variants, introduction of the wild-type gene should correct the cellular phenotype, unless the mutation is dominant-negative. If the mutant allele is dominant by haploinsufficiency, it can be rescued by overexpression of the wild-type gene as well. For gain-of-function or dominant-negative mutations, knockdown, knockout, or correction of the mutant allele should correct the cellular phenotype.
2. *Introduction of the genetic variant(s) in independent cell lines should reproduce or mimic the cellular phenotype.* Overexpressing the mutant allele in a different system should reproduce the cellular phenotype observed in patient primary cells. For example, loss-of-function mutations due to loss-of-expression must be confirmed by overexpressing the mutant allele(s) and confirming absent expression, and negative dominance must be established by co-transfecting the mutant and wild-type alleles into cells deficient for the gene product. As overexpression can alter the function of a gene, alternatively or additionally knockdown or knockout of the wild-type gene, or introduction of knock-in mutations in control cells, should reproduce the cellular phenotype.
3. If no relevant cellular phenotype can be found, the disease-causing effect of a genotype can be proved by an animal model, under the condition that it recapitulates the whole-organism phenotype of the patient *in vivo*. This means that the phenotype of the mutant animal must mimic the molecular, cellular and clinical phenotype of the patient. Mice are the most commonly used animal models for studying human immune disease [130]. Although the percentage of human genes without any homolog currently detectable in the mouse genome has been estimated to be less than 1% [131], there is always the possibility that

no mouse homolog of the gene of interest exists and that a different animal model must be used. Different techniques for manipulating the genome of animal models such as targeted mutagenesis or transgenesis have been developed [132], however, since animal models are not used in this work, they will not be further discussed.

RESULTS

By using the earlier stated approach, disease-causing variants were identified in two genes that were not known to be involved in severe early-onset immune pathology at the time of discovery, namely in *IFIH1* and *CECR1*. A *de novo* gain-of-function mutation in *IFIH1* was found in a 16-year-old girl with severe early-onset and refractory systemic lupus erythematosus, selective IgA-deficiency and mild lower limb spasticity without neuroradiological manifestations. These results were published in Arthritis & Rheumatology. Recessive loss-of-function mutations in *CECR1* were found in a 9-year-old boy with Castleman's-like disease and in two siblings with combined immunodeficiency, lymphoproliferation, autoimmunity and vasculopathy. These results were published as two separate comments on the original articles by Zhou *et al*. and Navon Elkan *et al*. in New England Journal of Medicine. Subsequently, a detailed description of the results of the treatment of one sibling with allogenic HSCT was published in the Journal of Allergy and Clinical Immunology. While the corresponding comment in New England Journal of Medicine was not referenced in the latter article, the reviewers of the Journal of Allergy and Clinical Immunology were made aware of its existence and content, and consented to the inclusion of these results in a more detailed paper. Additionally, compound heterozygous mutations in *STAT2* were found in two siblings who suffered from severe viral illness. STAT2 deficiency was previously described in a consanguineous family [75] and in two siblings suffering from febrile illness following live measles vaccine and of whom one went on to develop opsoclonus-myoclonus syndrome [133]. Identification of STAT2 deficiency in this third unrelated family strengthens this data. These results were submitted and are currently in review in the Journal of Allergy and Clinical Immunology. Each of the following chapters will be dedicated to one gene.

1. Chapter 1: IFIH1

Brief Report: *IFIH1* mutation causes systemic lupus erythematosus with selective IgA-deficiency

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Study design

The study was designed by Lien Van Eyck, Francis de Zegher, Carine Wouters and Adrian Liston.

Generation and analysis of data

The clinical data was collected by Lien Van Eyck with help from Lien De Somer, Francis de Zegher and Carine Wouters (Table 1). Identification of the disease-causing variant in *IFIH1* by whole-exome sequencing (Genomics Core Facility) and verification by Sanger sequencing was done by Lien Van Eyck (Figure 2A). Immunoprofiling on peripheral blood mononuclear cells was performed by Lien Van Eyck, with help from Diana Pombal (Figure 1). Measurement of IFN-α was performed by Lien Van Eyck, with help from Leen Moens and Glynis Frans (Figure 2B). Measurement of IFIH1 expression by RT-qPCR was done by Lien Van Eyck (Figure 2C). All data was analysed by Lien Van Eyck, Carine Wouters and Adrian Liston, with help from Simon Börnschein and Stéphanie Humblet-Baron for the analysis of the flow cytometry data.

Manuscript writing

Lien Van Eyck, Carine Wouters and Adrian Liston co-wrote the manuscript, which Xavier Bossuyt helped to edit.

Conflicts of interest

Lien Van Eyck, Simon Börnschein, Stéphanie Humblet-Baron and Leen Moens are funded by a research grant from Fonds Wetenschappelijk Onderzoek - Vlaanderen. Carine Wouters is funded by a research grant from University Hospitals Leuven and an unrestricted research grant from GSK immunoinflammation. Diana Pombal is employed by Vlaams instituut Biotechnologie. Glynis Frans is funded by a G.O.A. grant of University of Leuven. Xavier Bossuyt has received research support from the Research Council of University of Leuven. Adrian Liston is funded by Vlaams instituut Biotechnologie and by a European Research Council grant (IMMUNO). The spouse of A. Liston is an ex-employee of UCB Pharma. Other participants in the project declare no conflicts of interest.

Brief Report: *IFIH1* mutation causes systemic lupus erythematosus with selective IgA-deficiency

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ABSTRACT

Objective. To identify the underlying genetic defect in a 16-year-old girl with severe early-onset and refractory systemic lupus erythematosus (SLE), IgA deficiency, and mild lower limb spasticity without neuroradiologic manifestations.

Methods. Whole-exome sequencing and extensive immunologic analysis were performed on samples from the index patient.

Results. We identified a *de novo* p.R779H *IFIH1* gain-of-function mutation in a patient with severe early-onset SLE, selective IgA deficiency, and mild lower limb spasticity. The same mutation in *IFIH1* was recently identified in patients with Aicardi-Goutières syndrome, a rare neuroimmunologic disorder associated with elevated levels of type I interferon (IFN). IFN induced with helicase C domain 1 (IFIH1) functions as an intracellular innate immune receptor that senses viral nucleic acids and leads to the induction of type I IFN and proinflammatory cytokines. Despite systemic immunosuppressive treatment, disease activity persisted in the patient and was associated with elevated serum levels of IFNα and up-regulation of IFIH1 itself.

Conclusion. This finding adds a new genetic causation for Mendelian lupus and greatly extends the disease spectrum associated with mutations in *IFIH1* (ranging from inflammatory encephalopathy to prototypic systemic autoimmune disease). This marked phenotypic heterogeneity, despite an identical mutation, demonstrates the importance of modifying factors in type I IFN–dependent pathologies caused by mutations in *IFIH1*.

Keywords: systemic lupus erythematosus, IgA-deficiency, *IFIH1*

Interferon induced with helicase C domain 1 (IFIH1), a member of the retinoic acid–inducible gene 1 (RIG-I)–like family of cytoplasmic RNA receptors, senses double-stranded RNA and mediates an anti-viral response by activating type I interferon signaling [1]. Recently, gain-of-function mutations in *IFIH1* were identified in patients with Aicardi-Goutières syndrome (AGS) [2], a rare neuroimmunologic disorder associated with elevated levels of type I interferon (IFN) and characterized by leucoencephalopathy, brain atrophy and intracranial calcifications leading to profound intellectual disability, spasticity and dystonia [3]. The same *IFIH1* mutation was also identified in a patient with spastic paraplegia with normal results on neuroimaging and normal cognitive function [4].

Systemic lupus erythematosus (SLE) is a systemic multi-organ autoimmune disease that is associated with elevated levels of type I interferon [5]. Patients with monogenic causes of SLE are thought to comprise ~1% of the adult SLE cohort, with greater prevalence among patients with severe early-onset disease [6]. Known genetic defects associated with SLE include mutations in *DNASE1L3, DNASE1, PRKCD, TREX1* and *SAMHD1* [6]. Mutations in *TREX1* and *SAMHD1* are also found in AGS, demonstrating genetic associations between AGS and SLE [6]. In genome-wide association studies (GWAS) common polymorphisms in *IFIH1* have been identified as risk factors for SLE [7], type I diabetes mellitus [8] and selective IgA-deficiency [9].

PATIENT AND METHODS

The study was approved by the Ethics Committee of UZ Leuven, Belgium, and written informed consent was obtained from the parents of the patient and the age- matched healthy individuals. The study was performed in accordance with the modified version of the Declaration of Helsinki.

Clinical features of the index patient. The patient, a 16-year-old girl of European Belgian ancestry, initially presented with frequent respiratory infections before the age of 1 year. Immunologic testing revealed selective IgA deficiency. By the age of 2.5 years she developed lower limb spasticity, without cognitive or developmental impairment. Magnetic resonance imaging (MRI) of the brain and spine showed no abnormalities. At

Table 1: Blood analysis results in a SLE patient with a gain-of-function mutation in *IFIH1*.

	Age			
	8 years	15.3 years	16.2 years	16.8 years
WBC count, x 10^9 U/L (4.5 – 13.5)	5.8	5.01	7.44	7.86
Neutrophil count, , x 10^9 U/L (2.0 – 8.0)	3.6	2.8	6.4	6.0
Lymphocyte count, x 10^9 U/L (1.5 – 6.5)	1.4	1.7	0.9	1.1
Hemoglobin, g/dL (12 - 16)	10.6	11.9	11.7	9.9
Thrombocyte count, x 10^9 U/L (150 - 450)	108	243	297	294
Erythrocyte sedimentation rate, mm/h	74	29	11	8
C-reactive protein, mg/L	NA	<0.3	<0.3	<0.3
C3, g/L (0.79 - 1.52)	0.87	0.60	0.93	0.81
C3d, % (≤ 2.4)	4.6	12.2	3.0	2.3
C4, g/L (0.16 - 0.38)	0.1	NA	0.11	0.13
IgG, g/L (5.58 - 12.54)	16.9	18.9	10.3	9.36
IgA, g/L (0.13 - 1.08)	<0.07	NA	NA	NA
IgM, g/L (0.34 - 1.42)	1.44	NA	NA	NA
ANA titer †	>1/640	1/320	1/640	1/160
dsDNA (Farr), IU/mL	>100	>100	35.8	17.4
pANCA titer	1/640	NA	NA	NA
Anti-cardiolipin IgM, MPL U/ml (< 20)	78	NA	NA	NA
Anti-cardiolipin IgG, GPL U/mL (< 20)	8.0	26.7	4.4	NA
Lupus anticoagulans	Strongly positive	Strongly positive	Strongly positive	NA
Anti-thyroglobuline Abs, kU/L (≤ 200)	272	NA	NA	NA
Anti-thyroid peroxidase Abs, kU/L (≤ 100)	853	NA	NA	NA

SLE: systemic lupus erythematosus; WBC: white blood cell; NA: not available; ANA: antinuclear antibody; pANCA: perinuclear antineutrophil cytoplasmic antibody, Abs: antibodies. † By Farr immunoassay.

8 years old, the patient was diagnosed as having SLE with secondary antiphospholipid syndrome. Clinically, her disease manifested as arthritis, livedo rash, necrotizing cutaneous vasculitis, and deep venous thrombosis. Blood analysis demonstrated a marked inflammatory response, complement activation, and an abnormal autoantibody profile, including highly increased levels of anti–double-stranded DNA, anticardiolipin, and antithyroid antibodies (Table 1). Single photon–emission computed tomography, computed tomography, and MRI of the brain showed no abnormalities. Despite systemic immunosuppressive treatment, persistently increased levels of circulating autoantibodies and complement activation remained, and attempts at decreasing immunosuppressive medication were associated with disease flares. Spasticity remained confined to the lower limbs, cognitive functioning remained excellent, and results of all neuroradiologic examinations remained normal.

Whole-exome sequencing. We performed whole-exome sequencing on genomic DNA from the patient and the patient's unaffected mother. Genomic DNA samples were prepared from heparinized peripheral blood using a QIAamp DNA Blood Midi kit (Qiagen). Exome sequence libraries were prepared using a SeqCap EZ Human Exome Library version 3.0 kit (Roche NimbleGen). Paired-end sequencing was performed on an Illumina HiSeq2000 (Genomics Core Facility). Burrows-Wheeler Aligner software was used to align the sequence reads to Human Reference Genome Build hg19. GATK Unified Genotyper was used to identify single nucleotide variants and insertions/deletions. ANNOVAR software was used for annotation.

Sanger method of sequencing. The region of interest in exon 12 of *IFIH1* was sequenced using the primers 5'-CTTTCTTCCTCTGGAGTCACCCATC-3' and 5'-CAGACCTTCTTCTGCCACTGTGG-3'. Sanger sequencing was performed on an ABI 3730xl Genetic Analyzer (Applied Biosystems) at the LGC Genomics Facility in Berlin, Germany. Sequencing data were analyzed using CLC Main Workbench 6.9.1 (CLC Bio).

Flow cytometry. Peripheral blood mononuclear cells (PBMCs) were isolated from the heparinized blood of subjects using a lymphocyte separation medium (MP Biomedicals) and frozen in 10% DMSO (Sigma). Thawed cells were stained with antibodies against CD11c (3.9), CD3 (SK7), CD4 (RPA-T4), CD8a (RPA-T8), CD19

(HIB19), CD45RA (HI100), CD56 (MEM188), HLA–DR (LN3), IFNg (4S.B3), interleukin-17 (IL-17) (eBio64DEC17), IL-2 (MQ1-17H12), CD31 (WM-59), CCR7 (3D12), CD27 (O323), IgE (IgE21), CD24 (eBioSN3, SN3 A5-2 H10), CD38 (HIT2), g=d T cell receptor (B1.1), CD56 (MEM188), CD14 (61D3), CD123 (6H6), and IL-4 (8D4-8) (all from eBioscience), FoxP3 (206D) and IgM (MHM-88) (both from BioLegend), and CXCR5 (IgG23) (R&D Systems). For cytokine staining, T cells were stimulated ex vivo for 5 hours in 50 ng/ml phorbol 12-myristate 13-acetate (PMA) and 500 ng/ml ionomycin (both from Sigma) in the presence of GolgiStop (BD Biosciences) before staining. Prior to intracellular staining, cells were surface stained as described, fixed, and permeabilized using a fixation/permeabilization buffer (eBioscience) for FoxP3 staining or using Cytofix/Cytoperm (BD Biosciences) for other intracellular staining. All data were acquired on a BDFACSCanto II and analyzed with FlowJo (Tree Star).

Real-time quantitative polymerase chain reaction (RT-qPCR). Total RNA was isolated from PBMCs using an RNeasy Mini kit (Qiagen). Complementary DNA (cDNA) was synthesized by reverse transcription using a SuperScript III First-Strand Synthesis System (Life Technologies). The cDNA was analyzed by real-time quantitative PCR. An appropriate amount of the cDNA was mixed with Fast SYBR Green Master Mix (Applied Biosystems) supplemented with gene-specific primers: for *IFIH1*, 5'-CATATGCGCTTTCCCAGTG-3' (forward) and 5'-TGAGCATACTCCTCTGGTTTCA-3' (reverse); for *β-actin*, 5'-CTGGGACGACATGGAGAAAA-3' (forward) and 5'-AAGGAAGGCTGGAAGAGTGC-3' (reverse); for *GAPDH*, 5'-AGAAGGCTGGG GCTCATTTG-3' (forward) and 5'-GCATCAGCAGAGGGGGCAGA-3' (reverse); and for *HPRT*, 5'-GTAGCCCTCTGTGTGCTCAAGG-3' (forward) and 5'-GGCTTATATCCAACACTTCGTGGGG-3' (reverse). Real-time quantitative PCR analysis was performed on a StepOnePlus realtime PCR system (ABI). The thermal cycling protocol was 1 cycle of 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. The resultant PCR products were analyzed with ABI 7500 software (Applied Biosystems). Gene expression was analyzed with the 2–ΔΔCt method [10], and all quantifications were normalized to the average of the level of *β-*

actin, *GAPDH*, and *HPRT*. Experiments were performed in triplicate and repeated twice.

RESULTS

Alterations in the peripheral immune system. Extensive profiling of the patient's peripheral immune cells was performed at age 15 years, when the patient's disease was clinically stable and treatment was limited to mycophenolate mofetil. Of the major mononuclear leukocyte cell types surveyed, natural killer cell percentages were decreased (data not shown), percentages of T cells were reduced (especially among CD4+ T cells), and CD19+ B cell percentages were increased compared to healthy age-matched individuals (Figure 1A). Within the CD19+ B lymphocyte population, plasmablast levels were elevated and an excess of CD27+ memory B cells (the majority of which were IgM+IgD+) was found (Figure 1B), whereas the percentage of switched memory B cells was reduced. Within the CD4+ T lymphocyte population, the percentage of naive cells and antigen-experienced cells in the patient was comparable to that in healthy individuals, and cytokine production after stimulation with PMA/ionomycin appeared normal (Figures 1C and D). CD4+ follicular helper T (Tfh) cell numbers were relatively increased (Figure 1D). The percentage of Treg cells (CD4+FoxP3+ T cells) was also increased (Figure 1D); however, few of these cells were CD25high (data not shown). Within the CD8+ T lymphocyte population, we found an expansion of antigen-experienced cells (Figure 1C); however, cytokine production after stimulation with PMA/ionomycin was normal (data not shown).

Identification of a de novo mutation in *IFIH1* and *IFIH1* expression in PBMCs. Whole-exome sequencing was performed on genomic DNA from the patient and the patient's unaffected mother. After filtering out common polymorphisms, we identified a heterozygous c.G2336A mutation in *IFIH1*, leading to a p.R779H missense mutation. The mutation in the patient and the *de novo* status were confirmed by Sanger sequencing of DNA from the patient, both parents, and the patient's sister (Figure 2A). The same p.R779H missense mutation in *IFIH1* was recently described by Rice et al in 2 individuals. Only after extended treatment with high-dose corticosteroids (which was started because of a severely debilitating disease flare) did we see a reduction in serum

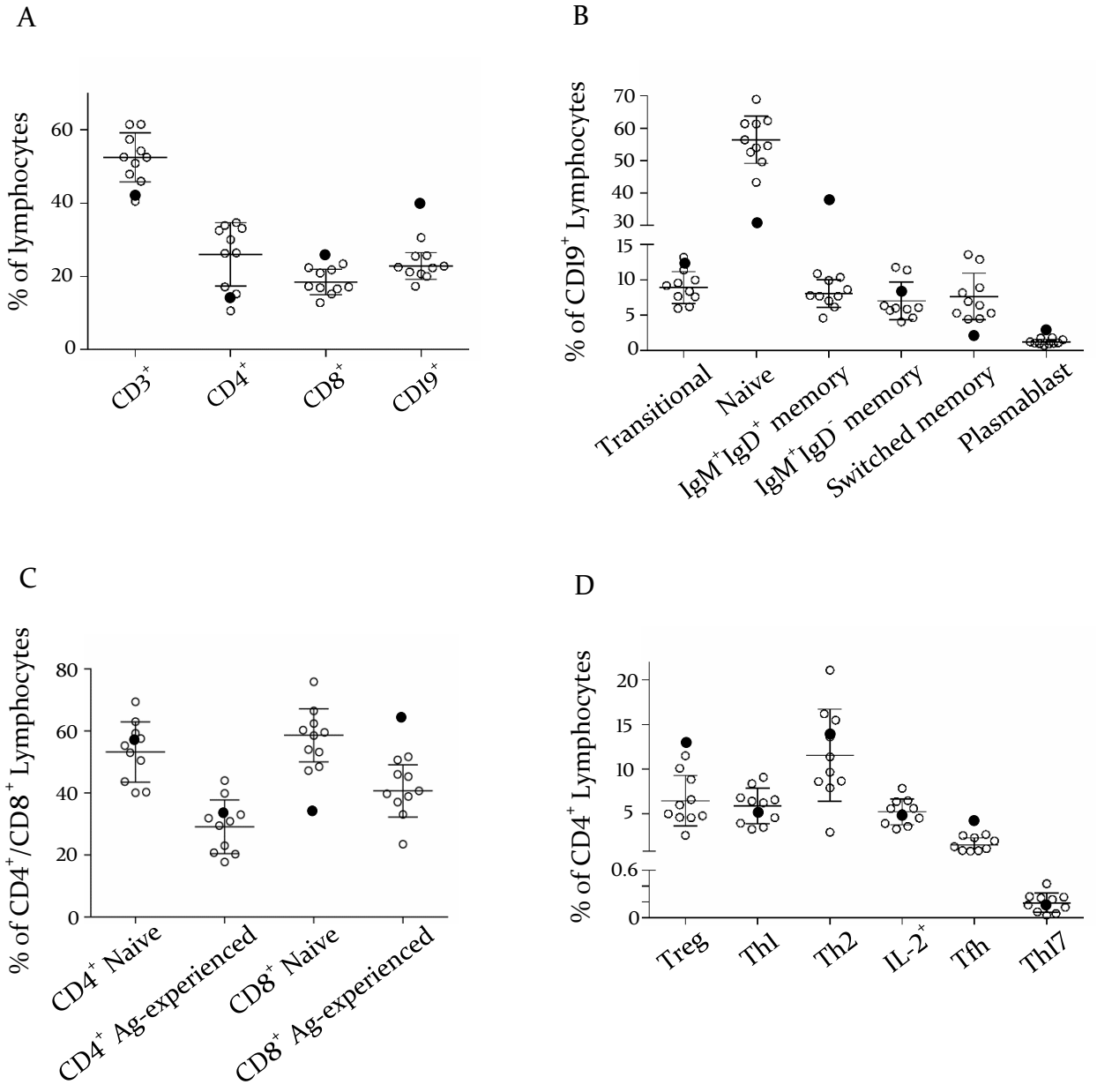

Figure 1. Immunologic profile of peripheral blood mononuclear cells from a systemic lupus erythematosus patient with a gain-of-function mutation in *IFIH1* (solid circles) as compared to healthy age-matched controls (open circles). Percentages of the major leukocyte subsets (A), CD19+ B lymphocyte subsets (B), CD4+ and CD8+ T lymphocyte subsets (C), and T helper cell lineages (D) were determined. Bars show the mean ± SD in the healthy controls. Ag: antigen; IL-2: interleukin-2; Tfh: follicular helper T cell.

levels of IFNα (Figure 2B). Subsequently, autoantibody titers were also reduced and complement activation was no longer present (Table 1), consistent with an IFNα-mediated mechanism of disease. As IFIH-1 is up-regulated by type I IFN (8), *IFIH1*

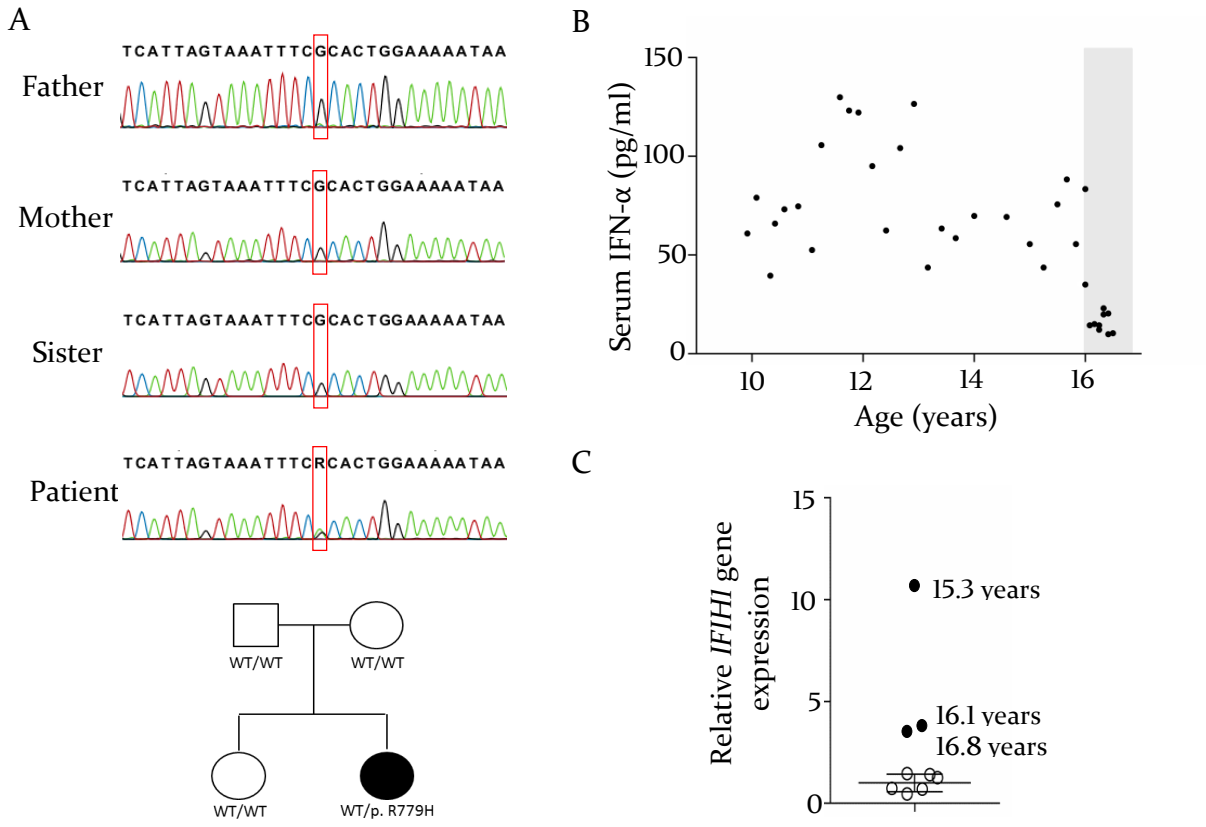

Figure 2. Confirmation of a *de novo* mutation in *IFIH1*, serum interferon-α (IFNα) levels, and *IFIH1* expression in peripheral blood mononuclear cells (PBMCs) from a systemic lupus erythematosus patient with a gain-of-function mutation in *IFIH1*. A, Sanger sequencing of the region of interest in exon 12 of *IFIH1* in samples from the patient and her unaffected family members (top). The pedigree of the affected patient (solid symbol) and her parents and sibling shows the *IFIH1* genotype (wild-type [WT; nonmutated] or p.R779H mutation) (bottom). B, Serum IFNα levels in the patient at various ages. Shaded bar indicates the period during which the patient was treated with corticosteroids. C, Relative *IFIH1* gene expression in PBMCs from the patient at 3 time points (solid circles) as compared to healthy age-matched controls (n = 6; open circles). Bars show the mean ± SD in the healthy controls.

expression in the patient's PBMCs was tested and found to be 10-fold up-regulated (Figure 2C). When serum levels of IFNα were reduced, the overexpression of *IFIH1* was reduced as well. These findings suggest that excessive activation of IFIH-1 is generated both from the gain-of-function mutation and also from the presence of a positive feedback loop around IFIH-1, leading to persistent production of IFNα and subsequent immune dysregulation.

DISCUSSION

We identified a *de novo IFIH1* mutation in a patient with elevated serum levels of IFNα, severe early-onset SLE, selective IgA deficiency, and mild lower limb spasticity without neuroradiologic manifestations. Strikingly, despite the common molecular etiology, the clinical presentation of this patient varied widely from that of other *IFIH1* gain-of-function patients (2,4). Although several cases in the cohort described by Rice et al manifested some immunologic abnormalities, the primary clinical manifestation was AGS. Conversely, the index patient described herein had no clinical features of AGS, with a clinical presentation dominated by early-onset SLE, IgA deficiency, and spastic paraplegia. In the study by Rice et al, 1 of the 2 AGS patients with the same p.R779H missense mutation developed chilblains at age 6 years and the other presented with generalized urticaria and mildly positive antinuclear antibodies at age 12 years (2). Strikingly, the father and paternal grandmother of the first patient carried the mutation but were asymptomatic. This indicates that the clinical phenotype associated with *IFIH1* mutations is diverse and includes an asymptomatic phenotype, isolated spastic paraplegia, severe inflammatory encephalopathy, and prototypic systemic autoimmune disease. This marked phenotypic heterogeneity demonstrates the importance of genetic, environmental, or stochastic modifying factors in type I interferonopathies caused by mutations in *IFIH1*.

In addition to the SLE manifestation, the patient described herein presented with selective IgA deficiency. We did not formally demonstrate whether selective IgA deficiency in the patient is due to the mutation in *IFIH1*; however, the association between selective IgA deficiency and SLE, as well as the finding of a common *IFIH1* polymorphism conferring risk in both diseases (7,9), indicates a shared genetic

predisposition between selective IgA deficiency and SLE through *IFIH1*. An intriguing possibility is that selective IgA deficiency may be the modifying factor that drove the interferonopathy toward the SLE phenotype in this patient. Selective IgA deficiency leads to a defective host defense against viral infections and an abnormal presentation of viral antigens. The possibility of exogenous viral-derived RNA playing a role in the pathogenesis of interferonopathies has been proposed as a possible explanation for the observed clinical non-penetrance (2). As serum IgA is thought to protect against autoimmunity by helping control inflammation, low levels of IgA or absent IgA may favor the development of autoimmunity and autoantibody production (11). It is therefore possible that selective IgA deficiency directed the interferonopathy in this patient toward the SLE phenotype. Further clinical investigation of patients with *IFIH1* mutations is necessary to determine the relative frequency of SLE and AGS, and whether selective IgA deficiency is disproportionally associated with one of the potential outcomes related to type I interferonopathies.

Immunologic assessment identified disturbances consistent with SLE, with increased levels of CD19+ B cells, CD27+ plasma cells, and a bias toward CD27+ IgM+IgD+ memory B cells. This immunologic signature has previously been described in patients with active SLE (12) and is indicative of a capacity for T cell–independent antibody production (13,14). Potentially contributing to a B cell–driven etiology was a relative increase in Tfh cell numbers and an increase in the CD4+CD25-FoxP3+ T cell population (a proposed dysfunctional Treg cell population) (15). This cell population can suppress T cell proliferation, but not IFNγ production, *in vitro* (15), so despite their increased proportion, the selective functional defect in these Treg cells might contribute to a failure to control autoimmune dysregulation. As these changes have been identified in other SLE patients (12,15,16), these results suggest that persistently elevated serum IFNα levels distort the immune system into an autoantibody-prone configuration. This mechanistic interpretation is consistent with our clinical observation of persistent inflammation and autoimmune activation despite immunosuppressive treatment. Serum levels of IFNα and, subsequently, autoantibodies were found to be reduced only after several months of treatment with high-dose corticosteroids. While this treatment has been effective, the IFNα-driven

nature of the disease process suggests that more targeted inhibition of IFIH-1 or IFNα would produce the same outcome with fewer side effects.

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2. Chapter 2: ADA2

Hematopoietic stem cell transplantation rescues the immunologic phenotype and prevents vasculopathy in patients with adenosine deaminase 2 deficiency

Lien Van Eyck, Michael S. Hershfield, Diana Pombal, Susan J. Kelly, Nancy J. Ganson, Leen Moens, Glynis Frans, Heidi Schaballie, Gert De Hertogh, James Dooley, Xavier Bossuyt, Carine Wouters, Adrian Liston*, Isabelle Meyts*

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Mutant ADA2 in vasculopathies

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Study design

The study was designed by Lien Van Eyck, Carine Wouters, Isabelle Meyts and Adrian Liston.

Generation and analysis of data

The clinical data was collected by Lien Van Eyck with help from Isabelle Meyts and Carine Wouters (Table E1, Figure E1 A). Histochemistry was performed by Gert De Hertogh (Figure E1 B). Identification of the disease-causing variants in ADA2 by whole-exome sequencing and verification by Sanger sequencing was done by Lien Van Eyck (Figure E2). Measurement of ADA2 plasma activity was done by Michael S. Hershfield, Susan J Kelly and Nancy J Ganson (Table 1 and 2). Immunoprofiling on peripheral blood mononuclear cells of P2 was performed by Lien Van Eyck, with help from Diana Pombal and James Dooley (Figure 1A-E). Immunoprofiling of P1 was performed by Gertjan Driessen of Erasmus MC Hospital Rotterdam (Table E2). Measurement of IL-6 was performed by Leen Moens with help from Lien Van Eyck, Glynis Frans and Heidi Schaballie (Figure 1F). All data was analysed by Lien Van Eyck, Carine Wouters, Isabelle Meyts and Adrian Liston.

Manuscript writing

Lien Van Eyck, Isabelle Meyts, Carine Wouters and Adrian Liston co-wrote the manuscripts, which Michael S. Hershfield and Xavier Bossuyt helped to edit.

Conflicts of interest

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Hematopoietic stem cell transplantation rescues the immunologic phenotype and prevents vasculopathy in patients with adenosine deaminase 2 deficiency

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SUMMARY

Adenosine deaminase 2 deficiency can present with immunodeficiency dominated by auto-immune cytopenia and lymphoproliferation. Hematopoietic stem cell transplantation restores adenosine deaminase 2 enzyme activity and represents a potentially curative treatment.

Keywords: adenosine deaminase 2 deficiency - immunodeficiency - hematopoietic stem cell transplantation

Abbreviations:

ADA	adenosine deaminase
CECR1	cat eye syndrome chromosome region, candidate 1
CID	combined immunodeficiency
CMV	cytomegalovirus
CT	computerized tomography
EBV	Epstein-Barr virus
GvHD	graft versus host disease
HHV	human herpes virus
HSCT	hematopoietic stem cell transplantation
HSV	herpes simpex virus
IVIG	intravenous immunoglobulin
MRI	magnetic resonance imaging
PBMC	peripheral blood mononuclear cells
PHA	phytohemagglutinin
PID	primary immunodeficiency
RSV	respiratory syncytial virus
VOD	veno-occlusive disease

To the Editor:

Recently, recessively inherited loss-of-function mutations in *CECR1* (cat eye syndrome chromosome region, candidate 1), which encodes adenosine deaminase 2 (ADA2), were identified in patients with a complex immunologic and vascular phenotype [1,2]. Possible mechanisms for this disorder are proinflammatory polarization and disturbed endothelial integrity [1,2]. Zhou *et al.* reported that aggressive systemic immunosuppressive treatment was not effective in controlling inflammation but hypothesized that hematopoietic stem cell transplantation (HSCT) might be curative given that bone marrow–derived monocytes and macrophages are the main source of secreted ADA2 [1]. Here we report on 2 related patients with homozygous p.Arg169Gln missense mutations in ADA2 located within the putative receptor-binding domain [3]. Our observations in these siblings demonstrate the clinical heterogeneity associated with ADA2 deficiency and show that HSCT can be an effective therapy. In the index patient the clinical course was dominated by autoimmunity and lymphoproliferation with a combined immunodeficiency–like phenotype, which prompted HSCT from a healthy sibling. Despite early complications, transplantation was successful both in rescuing the immunologic phenotype and in preventing vascular disease; at 5 years after HSCT, the patient remains off treatment.

The index patient (P1) was the second child of a father of Moroccan descent and a white mother. He was first admitted at age 6 months for complicated human *respiratory syncytial virus* infection. At this time, hypogammaglobulinemia was noted (see Table E1 in this article's Online Repository at www.jacionline.org). At age 12 months, P1 presented with fever, lymphadenitis, generalized lymphadenopathy, and hepatosplenomegaly. *Staphylococcus aureus* was cultured from the lymph nodes, and fever resolved within 24 hours of starting amoxicillin–clavulanic acid treatment. Pancytopenia, hypogammaglobulinemia, and the absence of specific antibodies were found (see Table E1). Results of blood PCRs for *Epstein–Barr virus* (EBV), *cytomegalovirus* (CMV), *human herpesvirus* (HHV) 6, HHV-8, and adenovirus were negative. However, adenovirus and norovirus were detected in the stool. Computed tomographic scans confirmed generalized lymphoproliferation with mediastinal and intra-abdominal lymphadenopathy and splenomegaly. Lymphoma was suspected, but

the results of lymph node biopsy and bone marrow examination were normal. Macrophage activation syndrome as the cause of the pancytopenia and lymphoproliferation was excluded based on serum markers (including soluble IL-2 receptor) and the absence of hemophagocytosis on bone marrow examination. A primary immune deficiency (PID) with predominant lymphoproliferation and autoimmunity was suspected, and known genetic causes were excluded. Prednisone (2 mg/kg) led to resolution of the thrombocytopenia and splenomegaly. However, attempts to taper led to a relapse of thrombocytopenia. Despite the addition of mycophenolate mofetil, sirolimus, tacrolimus, cyclosporine, and mercaptopurine, the cytopenia and lymphoproliferation persisted.

Because of growth failure secondary to chronic corticosteroid treatment, HSCT was considered at the age of 3 years. The patient's HLA-identical healthy elder brother was chosen as the donor. After conditioning with oral busulfan and cyclophosphamide, 7.5 X 10^6 CD34+ bone marrow–derived hematopoieticstem cells per kilogram were infused. Anti–graft-versus-host disease (GvHD) prophylaxis consisted of cyclosporine, whereas steroids were slowly tapered. Antiviral prophylaxis consisting of acyclovir and intravenous immunoglobulin (IVIG) administration and antifungal prophylaxis with fluconazole was added. The transplantation was complicated by late engraftment of neutrophils (day 26 <1.5 X 10^9/L) and persistent severe thrombocytopenia (<10 X 10^9/L) refractory to transfusion, although at day 28, whole blood chimerism was greater than 95%. At day 36, magnetic resonance imaging (MRI) of the brain, which was performed because of severe sudden-onset headache, identified a pineal gland hemorrhage (see Fig E1, A, in this article's Online Repository at www.jacionline.org). The thrombocyte level was 2 X 10^9/L but increased to greater than 50 X 10^9/L at day 40 after 2 infusions of rituximab. Veno-occlusive disease (VOD) was diagnosed according to the Seattle criteria at day 60 and was accompanied by a relapse of thrombocytopenia. VOD responded well to fluid restriction. Platelet levels of greater than 100 X 10^9/L were reached at day 111. Adenovirus reactivation was found at day 40, with accompanying intestinal GvHD grade III, which responded to corticosteroids. Cyclosporine was stopped at day 150. IVIG was discontinued at day 180. Immunoreconstitution at day 360 was excellent, including normal antibody levels, normal numbers of B- and T-

lymphocytes, and normal T-cell proliferation in response to PHA. Moreover, response to polysaccharide vaccine was normal (data not shown). Five years after transplantation, P1 is clinically well and off all medication. No more lymphoproliferation has occurred, and the most recent MRI of the brain 5 years after HSCT did not show any signs of acute or chronic small infarcts.

Two years after transplantation of P1, his younger brother (P2) presented at age 5 months with profound Coombs (2) anemia (hemoglobin, 2 g/dL), which was attributed to PCR-verified HHV-6–associated erythroblastopenia. At this time, immunologic analysis of P2 was normal. Several episodes of PCR-verified facial *herpes simplex virus* infection followed. At age 23 months, P2 was admitted with abdominal pain and ileus refractory to conservative treatment. He had generalized lymphadenopathy and hepatosplenomegaly, as well as hypogammaglobulinemia and intermittent lymphopenia and neutropenia (see Table E1). Results of blood polyomavirus PCR were positive. Bone marrow examination was normal. Partial enterectomy was performed; biopsy showed an atypical ulcerative bowel disease devoid of plasma cells (see Fig E1, B), as can be seen in patients with common variable immunodeficiency [4]. No CMV, EBV, herpes simplex virus, HHV-6, polyomavirus, or adenovirus could be detected in the biopsy specimen, and no signs of vasculitis could be observed in the entire surgical specimen. Obstruction persisted despite aggressive systemic immunosuppressive treatment and was only relieved after treatment with sirolimus. At this time, IVIG was started, and sirolimus was slowly tapered without clinical relapse. Subsequently, P2 did not receive any immunosuppressive treatment for a period of 13 months but was solely treated with IVIG. At 3.5 years of age, P2 had neurologic manifestations in the form of 2 episodes of acute loss of balance in the absence of fever or signs of systemic inflammation on blood analysis, Repeated MRI of the brain did not reveal any lesions compatible with ischemic or hemorrhagic stroke. A transient ischemic attack (TIA) was suspected, and treatment with sirolimus was restarted.

Whole-exome sequencing was performed on the untreated patient (P2), the parents, and the healthy sibling (for details, see the Methods section in this article's Online Repository at www.jacionline.org). We hypothesized a recessive model of inheritance. After filtering out common polymorphisms, we identified a homozygous c.G506A

variant in *CECR1*, resulting in a p.Arg169Gln missense mutation in ADA2. Sanger sequencing on DNA obtained from the cheek swab of the patient who underwent transplantation confirmed that he was also homozygous for this variant. Both parents were carriers, whereas the sibling donor was homozygous for the wild-type form of *CECR1* (see Fig E2 in this article's Online Repository at www.jacionline.org). ADA2 enzyme activity in plasma (Table I) was essentially absent in P2, the patient who did not undergo transplantation, whereas in post-HSCT plasma from P1, ADA2 activity was comparable with that of his healthy donor and in the range for healthy control subjects. Both parents have intermediate plasma ADA2 activity. Of note, neither adenosine nor deoxyadenosine levels were increased (<0.4 mmol/L) in plasma of P2 (these levels have not been measured in previous patients). Both P1 and P2 had normal ADA1 activity in dried blood spots, and deoxyadenosine nucleotides were undetectable.

Table I: Plasma ADA2 activity in the affected pedigree

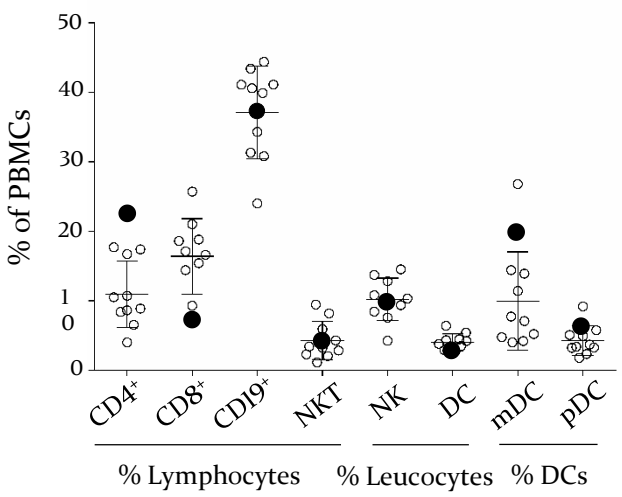
Sample	**Age (years)**	**Plasma ADA2 activity (mU/mL)**
Patient 1 post-HSCT	8	22.07
Patient 2	3	0.11
Healthy sibling (= HSCT donor)	10	19.14
Father	43	7.20
Mother	40	2.91
* Reference values for plasma ADA2 activity (mU/mL), mean ± SD (min-max)		
ADA2 Deficient (n = 4)		1.0 ± 0.4 (0.6 – 1.4)
ADA2 Carriers (n = 4)		4.9 ± 0.3 (4.6 – 5.3)
Control subjects (n = 5 + pooled human plasma)		14.0 ± 6.1 (4.8 – 21.3)

Although it has been speculated that the clinical consequences of ADA2 deficiency might be due to increased extracellular adenosine, our findings suggest this is not the case and that ADA2 actually has a minimal role compared with ADA1 in adenosine

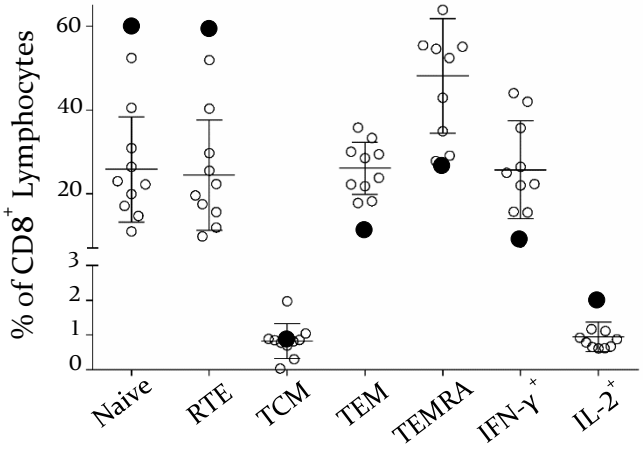
metabolism *in vivo*, which is consistent with the very different substrate affinities of the 2 ADA enzymes (see the Methods section in this article's Online Repository).

Because of the observed immunodeficiency, we performed extensive profiling of peripheral immune cells of P2 (for details, see the Methods section in this article's Online Repository). Of the major mononuclear leukocyte cell types surveyed, CD4+ T cell numbers were increased and CD8+ T cell numbers were reduced in P2 compared with those in healthy age-matched control subjects. B cell, natural killer cell, and dendritic cell numbers were within 1 SD of the mean of the healthy control subjects (Fig 1, A). Within the T lymphocyte population, we found defective T cell activation, with increased naive and low effector and memory subsets (Fig 1, B and D). Within the Th cell population, numbers of regulatory T cells were increased, whereas Th1, Th2, and follicular helper CD4+ T cell numbers were low (Fig 1, C). T cell proliferation in response to *Candida* species, tetanus, and PHA was normal (data not shown). Within the B lymphocyte population, naive B cell numbers were increased at the expense of memory and plasmablasts (Fig 1, E), which is suggestive of a defect in B lymphocyte differentiation or T cell provision of help. Limited immunoprofiling performed before HSCT showed similar findings in P1 (see Table E2 in this article's Online Repository at www.jacionline.org).

A



B



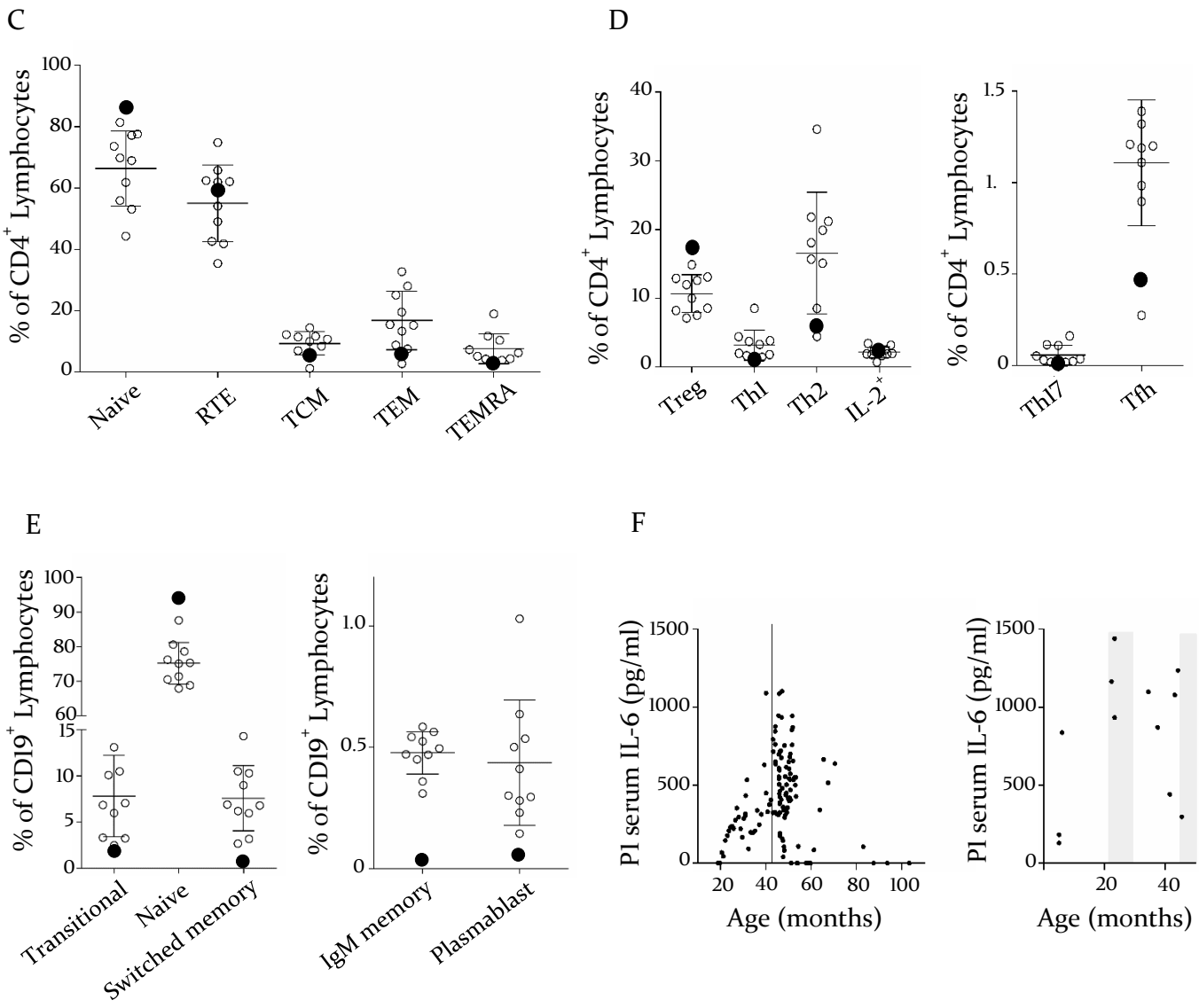


Figure 1. Serum IL-6 levels and immunoprofiling in ADA2-deficient patients. A, Major blood leukocyte subsets. B, CD4+ T lymphocyte subsets. C, Th cell lineages. D, CD8+ T lymphocyte subsets. E, B cell subsets. P2's values are shown as filled circles, and values of healthy age-matched control subjects are indicated by open circles. Means and SDs (error bars) shown exclude values for the patient. F, IL-6 levels in sera of P1 and P2. The vertical line indicates the moment of HSCT followed by pineal stroke in P1. The gray shading indicates the periods in which P2 was treated with sirolimus. DC, Dendritic cell; mDC, myeloid dendritic cell; NK, natural killer cell; NKT, natural killer T cell; pDC, plasmacytoid dendritic cell; RTE, recent thymic emigrant; TCM, central memory T cell; TEM, effector memory T cell; TEMRA, CD45RA-expressing effector memory T cell; Tfh, follicular T cell; Th17, IL-17–expressing helper T cell; Treg, regulatory T cell.

Because of the presence of severe inflammation in P1, serum IL-6 levels were measured from initial evaluation to last follow-up (Fig 1, F). IL-6 levels were persistently high before HSCT and before engraftment, but after HSCT IL-6 levels slowly decreased and were undetectable at 3 years post-HSCT. In P2 serum IL-6 levels were extremely increased, despite the absence of clinical signs of inflammation, with levels peaking at the time of bowel obstruction and at the time of the suspected TIAs. IL-6 was undetectable in the healthy sibling and in healthy control subjects. Moreover, TNF-α was not detectable in the serum of P1 and P2 at the time of the highest IL-6 levels. The immune profile of the other family members was normal (data not shown).Together, these data demonstrate a profound defect in T cell–dependent antibody-mediated responses and a failure to regulate normal inflammatory cytokine production in ADA2-deficient patients, adding to the previously identified function of ADA2 in *in vitro* stimulation of Th cells [5].

PIDs with autoimmunity and lymphoproliferation dominated the clinical image in our patients. The index patient P1 presented with persistent autoimmune pancytopenia and lymphoproliferation, whereas P2 had an episode of lymphoproliferation, bowel involvement, and 2 possible TIAs. Both patients only had fever during infectious episodes, and unlike previously reported patients, neither showed skin involvement or clear signs of vasculitis. P1 had a stroke as an apparent early complication of HSCT in the context of prolonged and severe thrombocytopenia. Only 3 years after initial presentation, P2 presented with 2 potential TIAs, although transient labyrinthitis caused by a viral infection could not be excluded. Therefore in retrospect vasculitis and inflammation might have been present at a subclinical level in both patients, but vasculopathy and inflammation did not dominate the clinical presentation, as is the case in the patients reported by Zhou *et al.* [1] and Elkan *et al.* [2]. Interestingly, serum IL-6 levels were increased in both patients in the absence of clinical and (routine) biochemical signs of inflammation. This suggests that ADA2 deficiency might lead to a subclinical state of inflammation. This phenotypic discrepancy cannot be explained entirely by *CECR1* genotype because the p.Arg169Gln mutation was previously observed in hemizygous and homozygous form [1,2]. The ADA2-deficient patients previously described had decreased serum immunoglobulin levels and enhanced B cell

apoptosis in vitro [1]. By contrast, our patients had abnormalities suggesting an *in vivo* defect in T cell activation and proliferation, corresponding to their increased susceptibility to viral infections and combined immunodeficiency–like phenotype. Taken together, these observations suggest that ADA2 deficiency has a more varied clinical phenotype than initially reported and that the diagnosis should be considered in cases of undiagnosed PID characterized by lymphoproliferation and autoimmunity, even in the absence of overt vasculopathy or inflammation.

As reported by Zhou *et al.* we found that treatment with a variety of immunosuppressive medications resulted in poor disease control in P1 [1]. However, both at the time of bowel obstruction and at the time of potential TIA, P2 seemed to respond well to sirolimus treatment. Sirolimus reduces M1 macrophage differentiation and IL-6 production [6]. Because ADA2 deficiency drives macrophages toward a more proinflammatory M1 profile [1], we present sirolimus as a potential therapeutic option to at least temporarily control inflammatory complications in ADA2-deficient patients. TNF-α was undetectable in the serum of our patients. However, this finding does not at all exclude a role for this cytokine in disease pathogenesis. Indeed, etanercept led to a significant response in all patients reported by Elkan *et al.*[1] and should therefore be considered as a potential treatment.

In the index patient P1 we successfully performed an allogeneic HSCT. At 5 years after HSCT, consecutive clinical and biochemical investigations in P1 have shown no signs of immunologic disorder and no additional strokes. This result supports the potential of HSCT as a long-term treatment strategy for ADA2 deficiency. However, caution is warranted because the HSCT procedure in P1 was characterized by severe early complications. Indeed, ADA2-deficient patients might present as high-risk candidates for HSCT. First, the inflammatory response associated with conditioning is superimposed on the inflammatory state intrinsic to ADA2 deficiency, which might negatively affect engraftment. Second, the compromised endothelial integrity observed in patients with ADA2 deficiency could predispose to development of VOD, a potentially fatal complication of HSCT. This combination of inflammation and endothelial injury might further increase the risk of stroke in the pre-engraftment and early post-engraftment phases [7] as observed in P1. It is reasonable to hypothesize that

ADA2-deficient patients might benefit from VOD prophylaxis with defibrotide, as well as from pretreatment with anti–IL-6 mAbs, rituximab, or both. Moreover, treatment with etanercept peri-HSCT could be considered in the context of ADA2 deficiency, especially given its usefulness in preventing and treating acute GvHD. However, given the underlying immunodeficiency, the risk of infection needs to be carefully balanced when using anti–IL-6 and anti–TNF-a mAbs.

Allogeneic HSCT restored normal plasma ADA2 activity in PI, which is consistent with bone marrow–derived monocytes and macrophages being the main sources of secreted ADA2. Whether ADA2 plays a role in other tissues and the effect of this on long-term prognosis remains unclear. A recent report on HSCT in a patient with ADA2 deficiency with a 9-year follow-up is promising and supports our findings [8]. However, it is plausible that the benefit from HSCT to our patient is entirely due to restoration of normal plasma ADA2 levels. If true, future treatment with exogenous ADA2 might provide an alternative therapy for ADA2 deficiency in patients in whom allogeneic HSCT is contraindicated.

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Online Repository

METHODS

The study was performed in accordance with the modified version of the Declaration of Helsinki. The study was approved by the Ethics Committee of UZ Leuven. Written informed consent was obtained before DNA isolation from blood of all family members and from cheek epithelium of the transplanted patient.

Functional assays

PBMCs were isolated from heparinized blood of patients, family members, and control subjects and analyzed by using flow cytometry, as previously described [E1]. Serum IL-6 levels were measured by means of ELISA, according to the manufacturer's instructions (BD Bioscience, San Jose, Calif).

Whole-exome sequencing. We performed whole-exome sequencing on the untreated patient and on the unaffected parents and sibling. Genomic DNA samples for whole-exome sequencing were prepared from heparinized peripheral blood by using the QIAamp DNA Blood Midi Kit (QIAGEN, Hilden, Germany). Exome sequence libraries were prepared with a SeqCap EZ Human Exome Library v3.0 kit (Roche NimbleGen, Madison, Wis). Paired-end sequencing was performed on the Illumina HiSeq2000 (Genomics Core Facility, University of Leuven, Leuven, Belgium). BWA software was used to align the sequence reads to the Human Reference Genome Build hg19. The GATK Unified Genotyper was used to identify single nucleotide variants and insertions/deletions. ANNOVAR was used for annotation.

Sanger sequencing. A somatic DNA sample of the patient undergoing transplantation was obtained from a cheek swab by using the GenElute Mammalian Genomic DNA Miniprep Kit (Sigma-Aldrich, St Louis, Mo). The region of interest in exon 2 of *CECR1* was sequenced with the primers 5'-GTTTGTACCAAGGGAGACACCTACC-3' and 5'-CTGGCTGGTGAGGAATGTCAC-3'. Sanger sequencing was performed on an ABI 3730 XL Genetic Analyzer (Applied Biosystems, Foster City, Calif) at the LGC Genomics Facility in Berlin, Germany. Sequencing data were analyzed by using DNADynamo (Blue Tractor Software, Llanfairfechan, United Kingdom).

Flow cytometry. PBMCs were isolated from heparinized blood of patients and control subjects by using lymphocyte separation medium (MP Biomedicals, Solon, Ohio) and frozen in 10% dimethyl sulfoxide (Sigma). Thawed cells were stained with antibodies (from eBioscience [San Diego, Calif], unless stated otherwise) against CD11c (3.9), CD3 (SK7), CD4 (RPA-T4), CD8a (RPA-T8), CD19 (HIB19), CD45RA (HI100), CD56 (MEM188), HLA-DR (LN3), forkhead box protein 3 (FOXP3; 206D; BioLegend, San Diego, Calif), IFN-g (4S.B3 IL-17, eBio64DEC17), IL-2 (MQ1-17H12), CXCR5 (IgG23; R&D Systems,Minneapolis,Minn), CD31 (WM-59), CCR7 (3D12), IgM (MHM-88, BioLegend), CD27 (O323), IgE (IgE21), CD24 (eBioSN3, SN3 A5-2 H10), CD38 (HIT2), gd T-cell receptor (B1.1), CD56 (MEM188), CD14 (61D3), CD123 (6H6), and IL-4 (8D4-8). For cytokine staining, T cells were stimulated *ex vivo* for 5 hours in 50 ng/mL phorbol 12-myristate 13-acetate (Sigma) and 500 ng/mL ionomycin (Sigma) in the presence of GolgiStop (BD Biosciences) before staining. Before intracellular staining, cells were first surface stained as described, fixed, and permeabilized with fixation/permeabilization buffer (eBioscience) for forkhead box protein 3 staining or Cytofix/Cytoperm (BD) for other intracellular stainings. All data were acquired on BD FACSCanto II and analyzed with FlowJo (Tree Star, Ashland, Ore).

ELISA for measurement of IL-6 levels in serum. An in-house validated ELISA was used based on a commercially available antibody pair (BD Biosciences).

Measurements of ADA1 and ADA2 activity in plasma. ADA2 activity in plasma was measured by using the HPLC method described by Zhou *et al.*[E2] ADA1 activity and concentrations of total adenosine and deoxyadenosine nucleotides in extracts of dried blood spots were measured, as previously described [E3,E4]. The concentrations of adenosine and deoxyadenosine in plasma were determined by means of HPLC analysis of a neutralized perchloric acid extract of plasma. In brief, 200 mL of plasma was acidified with 40 mL of 5 N perchloric acid and centrifuged, and the supernatant was neutralized with 3 N KOH and 1 M KHCO3. After centrifugation, 100 mL of the supernatant was analyzed on a C18 mBondapak column (Waters Corporation, Milford, Mass) by using 0.05 mol/L NH4H2PO4, 8% methanol, and 1% acetonitrile (pH 5.2; flow rate, 0.5 mL/min) as the mobile phase and monitoring absorbance at 260 and 280 nm with a diode array detector. The lower limit of quantitation for adenosine and

deoxyadenosine in this assay was 0.8 mmol/L; the lower limit of detection was taken as half the lower limit of quantitation or 0.4 mmol/L.

RESULTS

X-linked lymphoproliferative disease type I and II, Wiskott-Aldrich syndrome, autoimmune lymphoproliferative syndrome, ADA1 deficiency, purine nucleoside phosphorylase deficiency, and immune dysregulation–polyendocrinopathy–enteropathy–Xlinked syndrome were excluded by means of functional and genetic analyses.

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FIGURES

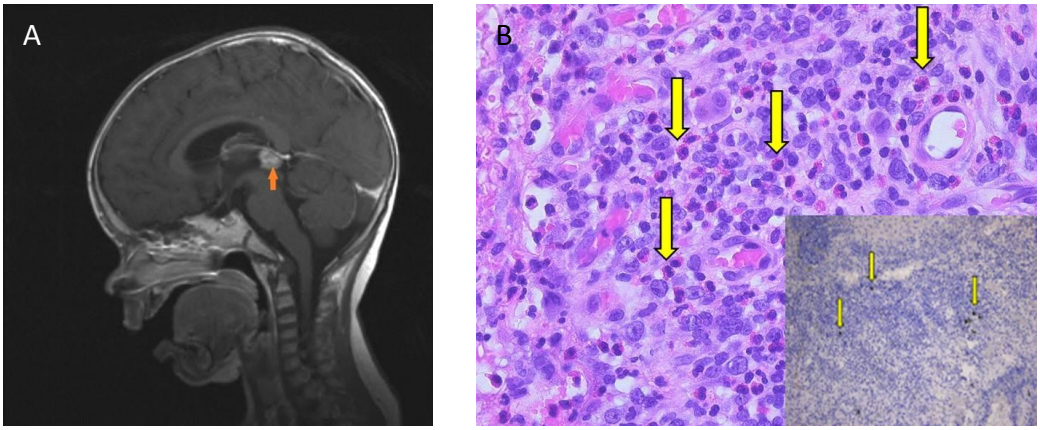

Figure E1: Vasculopathology and immunopathology in patients with ADA2-deficiency. A, Sagittal T1-weighted MRI of P1 showing pineal gland hemorrhage (arrow). B, Hematoxylin and eosin staining of jejunal ulceration in P2 showing chronic ulcer with predominant eosinophils (arrows), some neutrophils and lymphocytes and very few plasma cells. Plasma cells as stained by CD138 staining are indicated by arrows in the inlet.

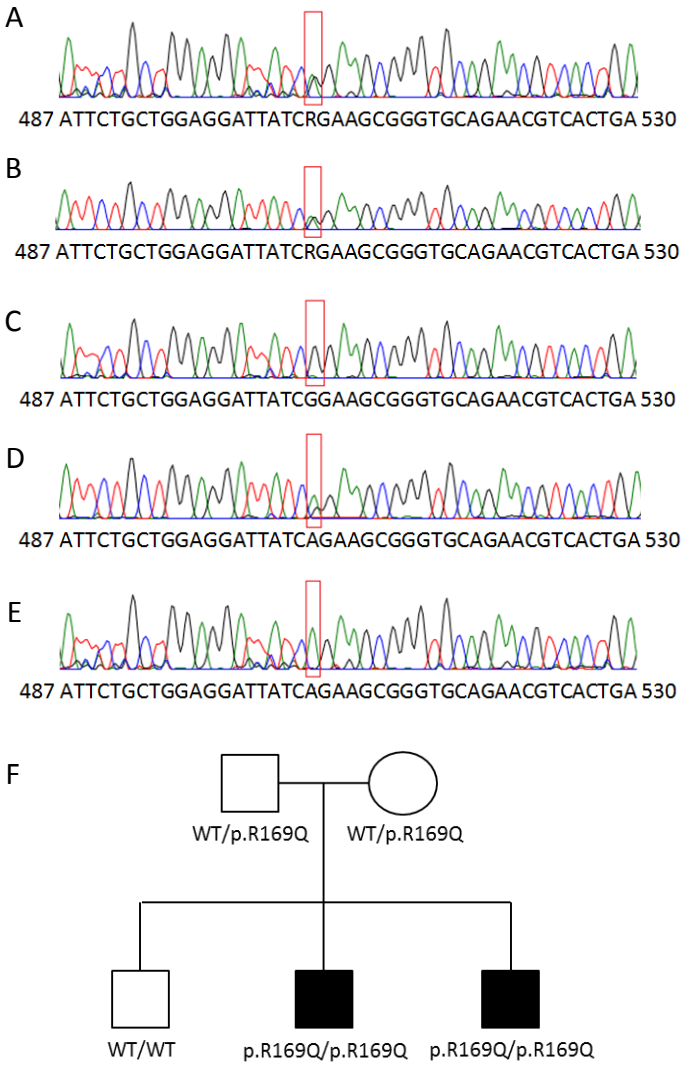

Figure E2: Familial inheritance of *CECR1* mutation. The region of interest in exon 2 of *CECR1* was sequenced by means of Sanger sequencing. A-E, Sequence reads for the father (Fig E2, A), mother (Fig E2, B), healthy sibling (HSCT donor; Fig E2, C), patient 1 after HSCT (chimerism accounts for presence of a minor G peak; Fig E2, D), and patient 2 (Fig E2, E). F, Family tree of the affected pedigree, indicating affected patients and *CECR1* genotype.

Table E1: Clinical presentation, laboratory values, and therapeutic history of ADA2-deficient patients

.	**Patient 1**	**Patient 2**
Clinical phenotype		
Clinical presentation	hypogammaglobulinemia, pancytopenia, lymphoproliferation	hypogammaglobulinemia, (intermittent) lymphopenia and neutropenia, lymphoproliferation
Viral infections confirmed by PCR	RSV,adenovirus, norovirus	HHV-6, HSV, polyomavirus
Stroke	hemorrhage in pineal gland	none
Laboratory values (obtained at initial clinical presentation)		
White blood cell count (kU/µL)	2.02	9.08
Neutrophil count (kU/µL)	0.3	6.4
Lymphocyte count (kU/µL)	1.0	1.7
Hemoglobin (g/dL)	8.8	9.7
Thrombocytes (kU/µL)	25	300
ALT (5-38 U/L)	44	15
AST (0-41 U/L)	64	6
IgG (3.02 - 9.85 g/L)	<1.00	2.77
IgA (0.13 - 1.08 g/L)	<0.07	0.11
IgM (0.26 - 1.60 g/L)	0.09	0.27
IgE (0-91 IU/mL)	<2	30
IgD (<10 U/mL)	0	0
ANA	negative	not determined
ANCA	negative	not determined
Thrombocyte autoantibodies	anti-gpIIB-IIIa Ab	not determined
Erythrocyte autoantibodies	anti-MNSl Ab	not determined
Lymphocyte count (kU/µl) (at moment of immunophenotyping)	0.39	3.0
Therapeutic history		
Immunosuppressive medication	CS, sirolimus, MMF, tacrolimus, ciclosporin, mercaptopurin	CS, azathioprine, sirolimus
Immunoglobulin substitution	yes (before HSCT)	yes
Allogenic HSCT	yes	no

ALT, Alanine aminotransferase; ANA, antinuclear antibody; ANCA, antineutrophil cytoplasmic antibody; AST, aspartate aminotransferase; HSV, herpes simplex virus; RSV, respiratory syncytial virus; CS, corticosteroids; MMF, mycophenolate mofetil.

Table E2: Relative frequencies of peripheral blood leukocyte populations in P1 before HSCT compared with those in healthy age-matched control subjects

Subset	**Defining surface markers**	**Patient (%)**	**Healthy volunteers**
			Range (min-max)
T cells	$CD3^+$	80.6	52.9-65.2
$CD4^+$ T cells	$CD4^+$ $CD8^-$	63.8	29.4-65.2
→ Treg	$CD25^+Foxp3^+$	10.0	-
$CD8^+$ T cells	$CD4^-$ $CD8^+$	0.83	17.6-23.2
B cells	$CD19^+$	5.56	11.8-30.4
→ transitional	$CD38^{high}CD24^{high}$	0.2	
→ naive	$CD27^-IgD^+$	97.8	
→ immature	$CD27^+IgD^+$	0.1	
→ switched memory	$CD27^+$IgD-	0.3	

CD3+, CD4+, and CD8+ T cells and CD19+ B cells are shown as percentages of total lymphocytes. Regulatory T (Treg) cells are shown as percentages of CD4+ T cells. B cell subsets are shown as percentages of CD19+ B cells. Foxp3, Forkhead box protein 3.

Mutant ADA2 in vasculopathies

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No potential conflict of interest relevant to this letter was reported.

To the Editor:

The studies by Zhou *et al.* and Navon Elkan *et al.* identified novel mutations in *CECR1*, encoding ADA2, as the cause of a syndrome including systemic vasculopathy and inflammation. We independently identified a homozygous p.Gly47Arg mutation in *CECR1* in a Jewish boy with a phenotype similar to that seen in Castleman's disease and found treatment with anti–interleukin-6 receptor antibody (tocilizumab) to be curative. The boy presented at 5 years of age with recurrent fevers, splenomegaly, generalized lymphadenopathies, increasing levels of acute-phase reactants, anemia, thrombocytosis, and polyclonal hyperimmunoglobulinemia. Whole-body imaging with 18F-fluorodeoxyglucose–positron-emission tomography–computed tomography (FDG-PET-CT) showed multiple lymph nodes with FDG avidity and increased splenic uptake. Tests for human herpesvirus 8 (HHV-8) were negative. Serum levels of interleukin-6 were highly elevated (180.5 pg per milliliter), and immunohistochemical analysis of a specimen obtained on lymph-node biopsy confirmed strong expression of interleukin-6. Treatment with tocilizumab resulted in a rapid, complete, and persistent suppression of clinical features and laboratory abnormalities.

In mice, loss-of-function adenosine deaminase has been reported to stimulate interleukin-6 induction through activation of the adenosine A2B receptor [1], suggesting a link between the loss of enzymatic ADA2 function and the increase in levels of interleukin-6 in this patient. Our results indicate that mutant ADA2 is a cause of interleukin-6–mediated lymphoproliferation and systemic inflammation and should be investigated as a cause of Castleman's disease.

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ADDITIONAL DATA

Table 2: Plasma ADA2 activity in the affected pedigree of a patient with Castleman's-like disease

Sample	**Age (years)**	**Plasma ADA2 activity (mU/mL)**
Patient	8	0.18
Father	62	3.75
Mother	44	3.65
* Reference values for plasma ADA2 activity (mU/mL), mean ± SD (min-max)		
ADA2 Deficient (n = 4)		1.0 ± 0.4 (0.6 – 1.4)
ADA2 Carriers (n = 4)		4.9 ± 0.3 (4.6 – 5.3)
Control subjects (n = 5 + pooled human plasma)		14.0 ± 6.1 (4.8 – 21.3)

Mutant ADA2 in vasculopathies

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No potential conflict of interest relevant to this letter was reported.

To the Editor:

Zhou *et al.* and Navon Elkan *et al.* identified novel mutations in *CECR1* as the cause of a syndrome that includes vasculopathy, inflammation, and immunodeficiency. The authors suggest that hematopoietic stem-cell transplantation (HSCT) should be explored as a possible treatment for this new primary immunodeficiency. We have independently identified two brothers with homozygous p.Arg169Gln mutations in *CECR1* and found HSCT to be curative. One boy presented at 13 months of age and the other at 5 months of age.

The older patient presented with combined immunodeficiency, cytopenia, and lymphoproliferation. He underwent immunosuppressive therapy and immunoglobulin substitution, but disease control was insufficient. He then underwent allogeneic HSCT at 3 years of age. A hemorrhagic stroke of the pineal gland developed 36 days after HSCT. During the next 5 years, this patient had complete resolution of the immunologic phenotype and no further occurrences of stroke. The younger patient presented with a similar phenotype but had a better response to immunosuppression and immunoglobulin substitution.

Plasma ADA2 activity was in the normal range in the HSCT recipient, but was absent in his brother (Hershfield M: personal communication). These results independently verify the *CECR1* mutation as a cause of novel primary immunodeficiency and further support the suitability of HSCT for the treatment of this condition.

3. Chapter 3: STAT2

STAT2 deficiency: a bona fide Primary Immunodeficiency

Leen Moens, Lien Van Eyck, Dirk Jochmans, Tania Mitera, Glynis Frans, Xavier Bossuyt, Patrick Matthys, Johan Neyts, Michael Ciancanelli, Shen-Yin Zhang, Rik Gijsbers, Jean-Laurent Casanova, Stephanie Boisson-Dupuis, Isabelle Meyts*, Adrian Liston*

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Submitted to JOURNAL OF ALLERGY AND CLINICAL IMMUNOLOGY
In revision

Scientific acknowledgements

Study design

The study was designed by Leen Moens. Lien Van Eyck, Jean-Laurent Casanova, Isabelle Meyts and Adrian Liston.

Generation and analysis of data

The clinical data was collected by Lien Van Eyck with help from Isabelle Meyts (Table E1 and E2). Identification of the disease-causing variants in STAT2 by whole-exome sequencing and verification by Sanger sequencing was done by Lien Van Eyck (Figure 1A). Expression and activation of STAT1 and STAT2 on primary fibroblasts was examined by Lien Van Eyck, with help of Leen Moens and Glynis Frans (Figure 1B and 1C). Measurement RT-qPCR of pregulation of ISGs in primary fibroblasts in response to IFN-α/γ was performed by Leen Moens (data not shown), upregulation in SV40-transformed fibroblasts was measured by Lien Van Eyck (Figure 2). EMSA and supershift assays on SV40-fibroblasts were performed by Stéphanie Boisson-Dupuis (data not shown). The pMCSV puro plasmid containing wild-type STAT2 was generated by Lien Van Eyck, wild-type STAT2 cDNA was obtained from Michael Ciancanelli. Generation of the STAT2 variants by site-directed mutagenesis was performed by Lien Van Eyck, transfection and immunoblotting for phosphorylated STAT2 was done by Leen Moens (Figure 3). Analysis of *STAT2* transcripts revealing multiple splicing variants was performed by Leen Moens (data not shown). Viral assays on primary fibroblasts were performed by Lien Van Eyck, with help from Dirk Jochmans and Tania Mitera (Figure 4). Viral assays on SV40-transformed fibroblasts were done by Lien Van Eyck. Generating of the retroviral vector encoding wild-type STAT2 and transduction of SV40-transformed fibroblast were done by Lien Van Eyck with help of Leen Moens, verifying STAT2 expression by Western Blot was done by Leen Moens (Figure 5). The lentivirus for transduction of primary fibroblasts will be generated by Rik Gijsbers, transduction of primary fibroblasts will be performed by Rik Gijsbers, viral assays on transduced primary fibroblasts will be performed by Leen Moens. Leen Moens, Lien Van Eyck, Jean-Laurent Casanova, Isabelle Meyts and Adrian Liston analysed the data.

Manuscript writing

Lien Van Eyck, Isabelle Meyts and Adrian Liston co-wrote the manuscript, which Jean-Laurent Casanova, Shen-Yin Zhang, Xavier Bossuyt, Patrick Matthys and Johan Neyts helped to edit.

Conflicts of interest

Leen Moens and Lien Van Eyck are funded by a research grant from Fonds Wetenschappelijk Onderzoek - Vlaanderen. Glynis Frans is funded by a G.O.A. grant of University of Leuven. Xavier Bossuyt has received research support from the Research Council of University of Leuven. Johan Neyts is supported by BELSPO (IUAP, Belvir). Jean-Laurent Casanova is supported by the St. Giles Foundation, Institut National de la Santé et de la Recherche Médicale (INSERM), University Paris Descartes and The Rockefeller University. Isabelle Meyts is supported by a KOF mandate of University of Leuven and by the Jeffrey Modell Foundation. Adrian Liston is funded by Vlaams instituut Biotechnologie and by a European Research Council grant (IMMUNO). The spouse of A. Liston is an ex-employee of UCB Pharma. Other participants in the project declare no conflicts of interest.

STAT2-deficiency: a bona fide Primary Immunodeficiency

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SUMMARY

STAT2-deficiency is associated with a profound failure of type I interferon signalling leading to a defect in antiviral immunity and potentially fatal viral illness.

Key words: STAT2-deficiency, type I interferon, viral illness

Abbreviations:

CSF	cerebral spinal fluid
EBV	Epstein–Barr virus
EMSA	electrophoretic mobility shift assay
F2A	FDMV 2A peptide
FDMV	foot-and-mouth disease virus
GAF	gamma-interferon activation factor
GAS	gamma-interferon activation site
HPIV3	human parainfluenza virus type 3
HSV	herpes simplex virus
IFN	interferon
IFNAR	IFN-α/β receptor
IgE	immunoglobulin E
IgG	immunoglobulin G
IRF9	interferon regulatory factor 9
ISG	IFN-stimulated genes
ISG15	IFN-stimulated gene 15
ISGF3	interferon-stimulated gene factor 3
ISRE	IFN-stimulated response element

IVIG	intravenous immunoglobulin
JAK	Janus kinase
MeV	measles virus
MMR	measles-mumps-rubella
MSCV	murine stem cell virus
MX1	MX Dynamin-Like GTPase 1
NK cell	natural killer cell
OAS1	2'-5'-oligoadenylate synthetase 1
PBMC	peripheral blood mononuclear cells
PME	primary measles encephalitis
RSV	respiratory syncytial virus
RT-qPCR	real-time quantitative polymerase chain reaction
SSPE	subacute sclerosing panencephalitis
STAT2	transducer and activator of transcription 2
SV40	Simian virus 40
TLR	Toll-like receptor
TYK2	tyrosine kinase 2
VSV	vesicular stomatitis virus

To the editor:

STAT2 deficiency has been proposed as the cause of unusually severe viral illness in infancy. The only reported cases of STAT2 deficiency consist of an extended family in which two siblings developed disseminated vaccine-strain measles following routine immunization, of whom the infant sibling died from a probable viral infection [1]. Strikingly, there was incomplete clinical penetrance in this kindred, giving rise to questions regarding the completeness of the STAT2 defect. Two other siblings with STAT2 deficiency were also reported to suffer from febrile illness following live measles vaccine; one went on to develop opsoclonus-myoclonus syndrome [2]. STAT2 deficiency was associated with a profound failure of type I interferon (IFN) signalling, which is thought to be responsible for the observed defect in antiviral immunity.

The IFN response is one of the most important innate antiviral defences. Three types of IFNs have been identified: IFN-α/β (type I), IFN-γ (type II) and IFN-λ (type III) [3]. These cytokines are secreted following pathogen exposure, by most cell types (types I and III) or by activated T and NK lymphocytes, in the context of an immune response (type II IFN). Binding of IFNs to their receptor on their target cells leads to activation of the Janus kinase signal transducer and activator of transcription (JAK-STAT) signalling pathways and subsequently induces expression of hundreds of diverse IFN-stimulated genes (ISGs), the products of which may have direct or indirect antiviral activity. In the canonical type I IFN signalling pathway (Figure 1), binding of IFN-α/β to the type I IFN receptor IFNAR activates the receptor-associated JAK1 and tyrosine kinase 2 (TYK2), who then phosphorylate and activate STAT1 and STAT2. Phosphorylated STAT1 and STAT2 associate with interferon regulatory factor 9 (IRF9) to form the heterotrimeric transcription factor interferon-stimulated gene factor 3 (ISGF3) that binds to IFN-stimulated response elements (ISREs) in the promotor region of IFN-α/β–stimulated genes. Although type III IFNs bind to a distinct receptor, activation of the same signalling pathway occurs resulting in a similar transcriptional profile as type I IFNs [4]. In contrast, binding of type II IFN to the IFN-γ receptor activates JAK1 and JAK2, leading to phosphorylation of STAT1 but not STAT2 [3]. Phosphorylated STAT1 homodimerizes and forms the gamma-interferon activation factor (GAF), a transcription factor that binds to gamma-interferon activation site

(GAS) in the regulatory regions of IFN-γ–induced genes. Type I IFN can also weakly activate GAF due to the phosphorylation of STAT1. While ISGF3 induces the expression of classical antiviral genes, GAF induces the expression of pro-inflammatory genes [3]. Additionally, several IRFs can induce certain ISGs as well, either after direct activation by viral infection or following IFN induction [5]. Most cell types can respond to type I IFN (IFN-β and multiple IFN-α subclasses) and IFN-γ, whereas type III IFN (IFN-λ1, -λ2 and - λ3) mostly targets hepatocytes and mucosal epithelial cells, hereby providing protection against the frequent viral attacks that are typical for barrier tissues [4].

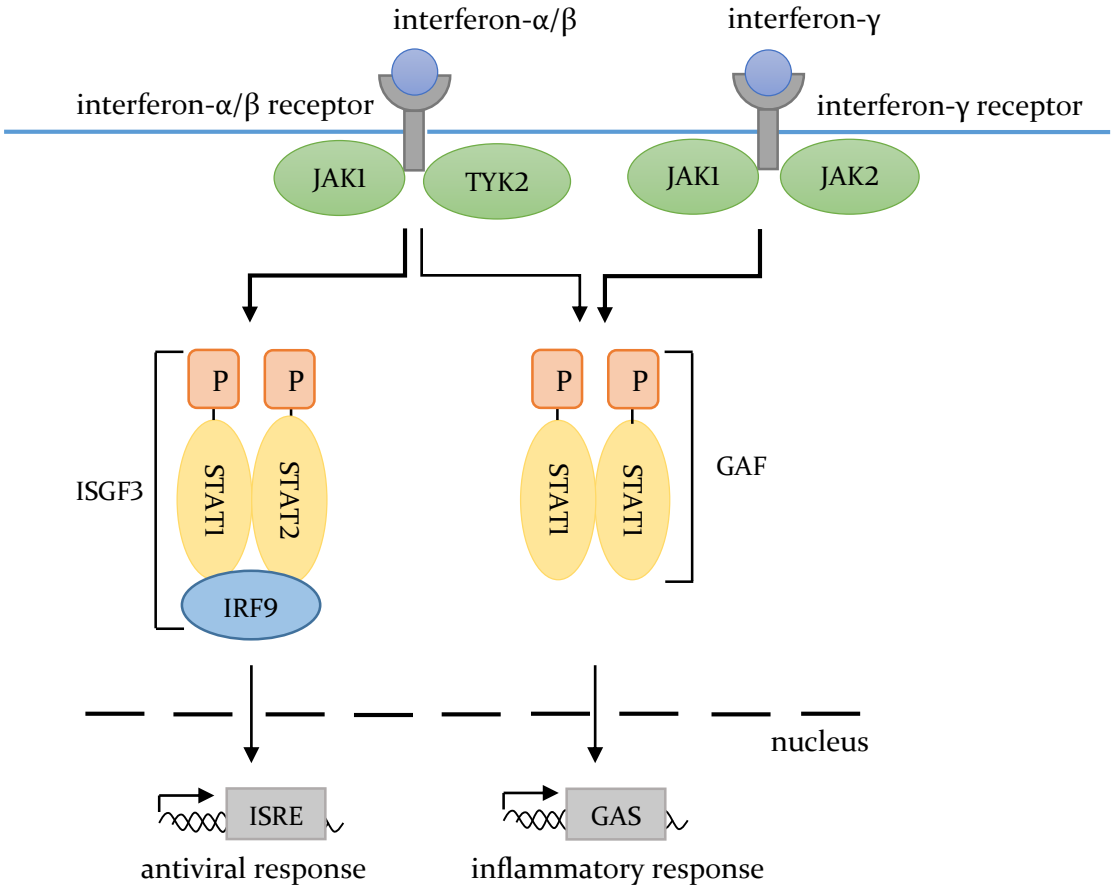


Figure 1: **The canonical type I and type II interferon pathway.**

Here we report on two siblings with compound heterozygous mutations in *STAT2* who suffer from severe viral illness and of whom one sibling succumbed at the age of 7 years from an infection with an unidentified agent.

RESULTS

Clinical and genetic features of the patients

Patient 1 (P1) was the second child of unrelated parents of Belgian European descent. The first 3 years of his life were characterized by frequent and severe viral infections often requiring hospital admission. At age 18 months he developed high fever 1 week after administration of the MMR-vaccine, with a morbilliform rash, conjunctivitis, lymphadenopathies and arthritis. He recovered with supportive care. Basic immunological screening was normal. Physical and mental development were normal apart from an increased tendency for dental caries. From the age of 4 years he had a relatively infection-free period, but at age 7 years he succumbed to an infection with an unidentified agent. Clinically he suffered from a mild febrile illness, but after 48h his condition suddenly deteriorated and he died of multiple organ failure in the context of diffuse intravascular coagulopathy. All cultures remained negative and no causal pathogen could be identified.

His younger sister P2 also suffered from frequent and severe viral infections in her first years of life, including severe and recurrent Varicella zoster infections, recurrent and disseminated Coxsackie infections, enteroviral meningitis and persistent cutaneous Human papillomavirus and Mollusca contagiosa. Infectious episodes were accompanied by moderate thrombopenia, lymphopenia, leucopenia and serological signs of immune dysregulation (including elevated IgG and IgE levels). Between infectious bouts, immunological screening could not demonstrate abnormalities in adaptive or innate immunity, with normal numbers of leucocyte subsets, normal lymphocyte proliferation tests, normal immunoglobulin levels and vaccine responses and normal activation of Toll-like receptor (TLR) pathways (Table E1). Physical and mental development were normal except for the same increased susceptibility to dental caries as P1. At age 18 months she developed disseminated measles following routine immunization, complicated by hepatitis and pneumonitis. At this time, she received a high dose of intravenous immunoglobulin (IVIG) (1 gram IVIG per kg body weight) due to her poor condition and the emergence of coagulopathy, after which she recovered and became afebrile within 24 hours. At age 2.5 years P2 suffered from a

severe primary infection with *Epstein–Barr virus* (EBV), associated with massive immune dysregulation that again responded well to high dose IVIG treatment. Macrophage activation syndrome was excluded based on international criteria. Over the next 3 years repeated measurements by PCR showed persistent EBV presence in both blood and cerebral spinal fluid (CSF), despite the presence of anti-EBV IgG (Table E2). From the age of 5 years the frequency and severity of viral infections decreased, but P2 still suffered from chronic lung disease with productive cough requiring chronic antibiotic treatment with azithromycine, inhalation steroids and daily respiratory physiotherapy. At age 9 years P2 still experiences several mild viral infections per year, mostly upper respiratory infections. She is off all medication.

Whole-exome sequencing was performed on P2, the parents and the younger healthy sibling (For detailed methods, see OR). After filtering out common polymorphisms, we found that P2 had compound heterozygous c.*G1576A* and c.*C1528T* variants in *STAT2*, resulting in a p.Gly522Arg missense mutation and a premature stop codon at amino acid 506 (p.Arg506X). Sanger sequencing on DNA obtained from a frozen bone marrow sample of the deceased brother P1 confirmed that he carried both variants as well. The p.Gly522Arg mutation was inherited from the mother, the premature stop codon p.Arg506X from the father. The healthy younger brother was homozygous for the wild type form of *STAT2* (Figure 2A); no DNA was available from the older healthy brother.

Assessment of the STAT2 defect

STAT1 and STAT2 expression and activation were examined in fibroblast and peripheral blood mononuclear cells (PBMC) of P2, the parents and the younger healthy brother as well as two unrelated controls (refer to the OR for Methods). Immunoblotting for STAT2 and phosphorylated STAT2 after stimulation with IFN-α (type I interferon) demonstrated absence of expression of both full length and truncated protein in P2, and reduced expression and phosphorylation in both parents compatible with their carrier status (Figure 2B). By contrast, STAT1 expression and phosphorylation after stimulation with IFN-γ (type II interferon) was normal (Figure 2C).

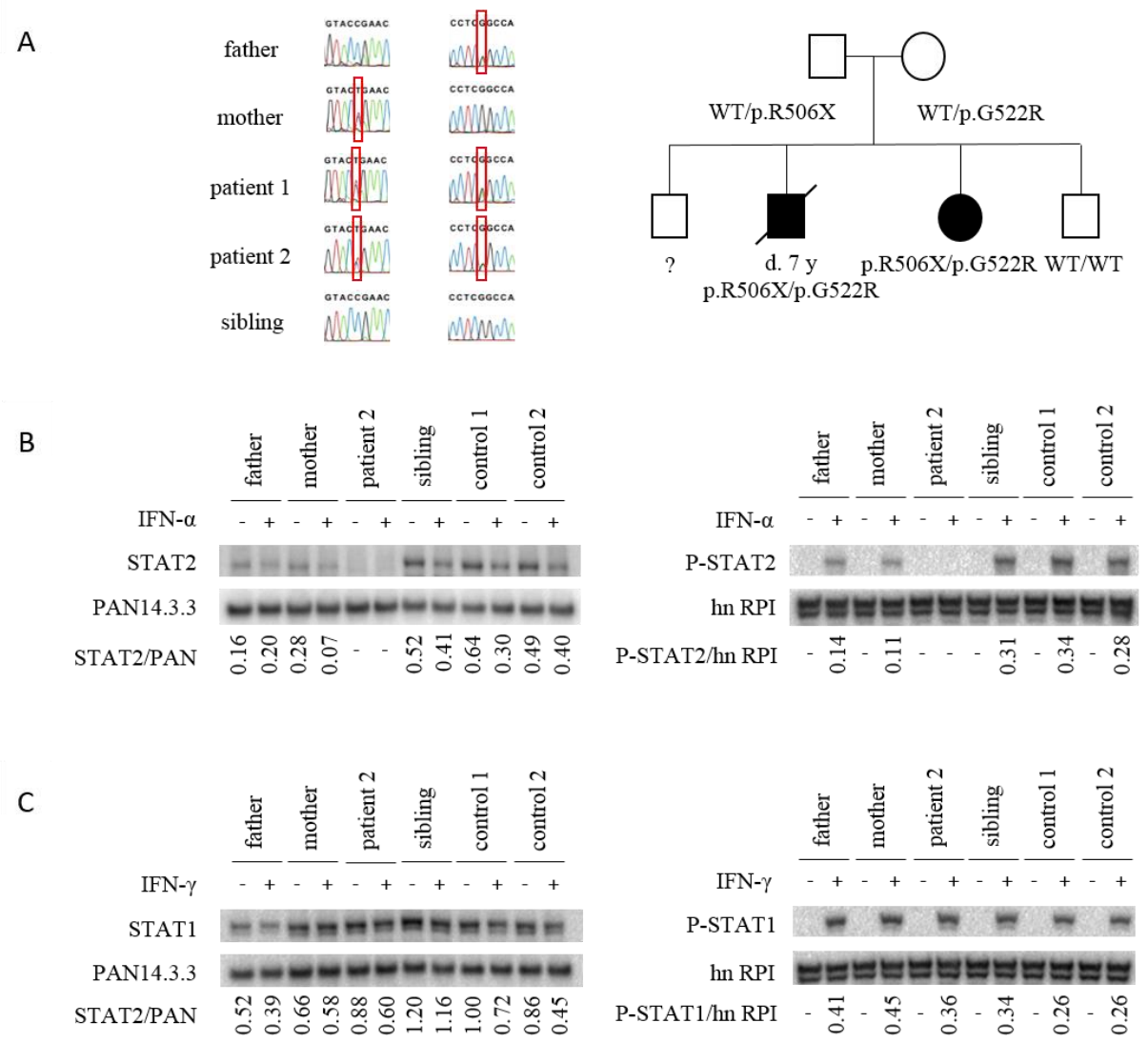


Figure 2**: Familial inheritance of *STAT2* mutations and expression of STAT1, STAT2, phosphorylated STAT1 and phosphorylated STAT2.** A) Family tree of the affected pedigree, indicating affected patients and *STAT2* genotype. B and C) Peripheral blood mononuclear cells (PBMC) and fibroblasts of all family members and 2 unrelated healthy controls were treated with IFN-α or IFN-γ at 10^3 Units per ml for 30 min or left untreated. STAT1 and STAT2 expression was verified on cytoplasmic protein extract of PBMC and expression of phosphorylated STAT1 and phosphorylated STAT2 was verified on nuclear protein extract of fibroblasts by Western Blot, expression of PAN14.3.3 and hn RPI respectively was assessed as loading control. WT, wild-type; IFN, interferon; STAT, signal transducer and activator of transcription; hn RPI, heterogeneous nuclear ribonucleoproteins I.

To further assess whether the STAT2 defect is complete or partial, upregulation of the IFN-stimulated genes (ISGs) *MX1*, *ISG15* and *OAS1* in response to IFN-α and IFN-γ was measured by RT-qPCR in primary fibroblasts of P2, her parents and her healthy sibling and in Simian virus 40 (SV40)-transformed fibroblasts of all family members and the STAT1-deficient individual (refer to the OR for Methods). While primary fibroblasts of all family members exhibited a similar response to IFN-γ and the fibroblasts of the parents and healthy sibling showed marked upregulation of the ISGs in response to IFN-α, fibroblasts of P2 failed to upregulate *MX1*, *ISG15* and *OAS1* in response to IFN-α (data not shown). In contrast, RT-qPCR on SV40-transformed fibroblasts of several

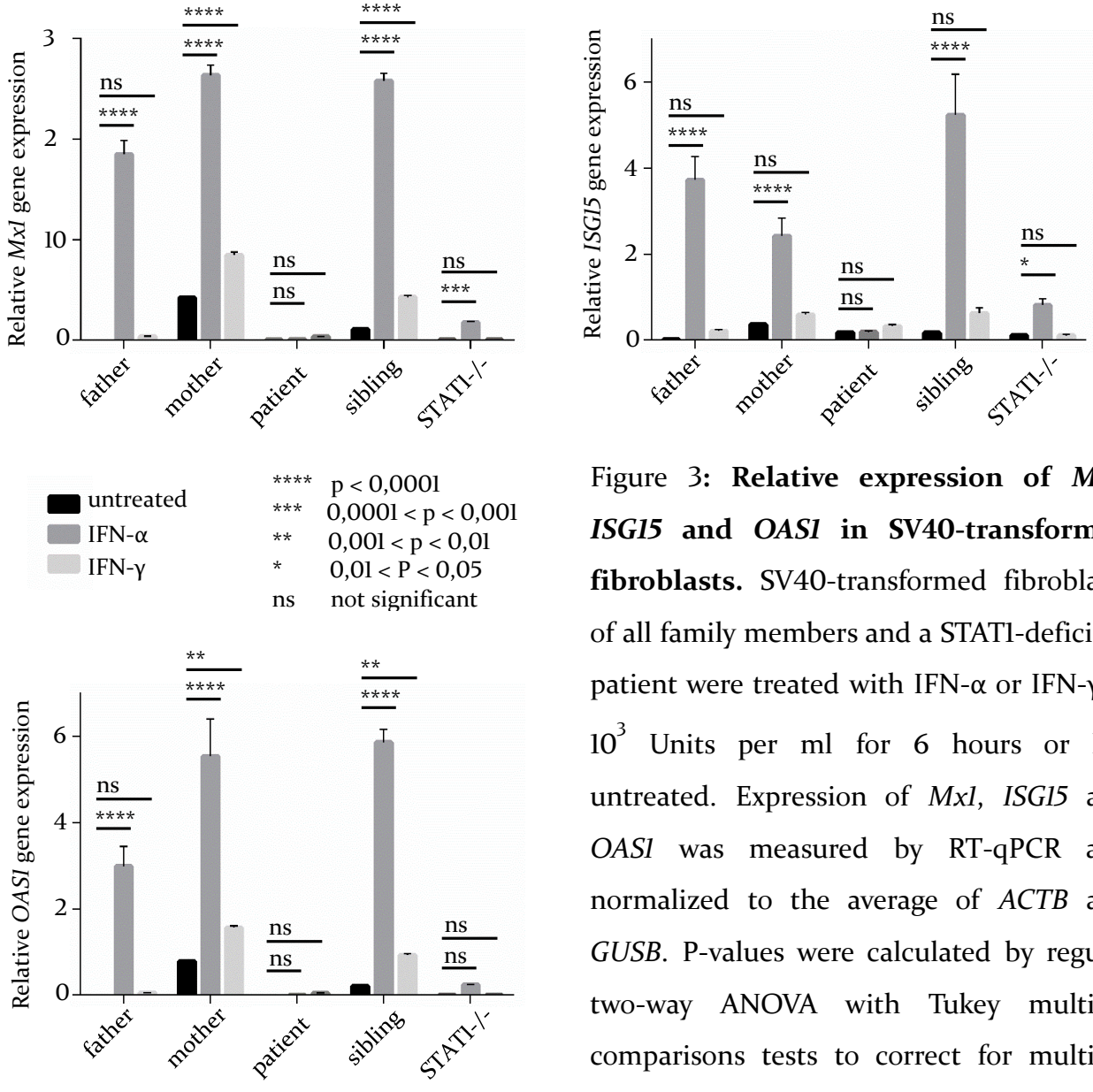


Figure 3**: Relative expression of *Mx1*, *ISG15* and *OAS1* in SV40-transformed fibroblasts.** SV40-transformed fibroblasts of all family members and a STAT1-deficient patient were treated with IFN-α or IFN-γ at 10^3 Units per ml for 6 hours or left untreated. Expression of *Mx1*, *ISG15* and *OAS1* was measured by RT-qPCR and normalized to the average of *ACTB* and *GUSB*. P-values were calculated by regular two-way ANOVA with Tukey multiple comparisons tests to correct for multiple comparisons.

family members demonstrated several abnormalities in the response to IFN-α and IFN-γ. Most strikingly, ISG upregulation was already present in SV40-transformed fibroblasts of the mother in untreated conditions, whereas ISG expression was very low in SV40-transformed fibroblasts of the father in untreated condition. SV40-fibroblasts of the father did upregulate ISGs in response to IFN-α but not significantly in response to IFN-γ, whereas SV40-fibroblasts of P2 failed to significantly upregulate *MX1*, *ISG15* and *OAS1* in response to IFN-α and IFN-γ (Figure 3). It has been demonstrated before that ectopic expression of SV40 large T antigen following immortalisation results in the induction of ISGs in human fibroblasts and confers an antiviral state [6], and these findings seem to confirm this.

Additionally, electrophoretic mobility shift assay (EMSA) was performed on SV40-transformed fibroblasts extracts of all family members (refer to the OR for Methods), to test for binding of the ISGF3-complex and GAF-complex to the ISRE-elements and GAS-elements respectively that are present in the promotor regions of ISGs, after stimulation with IFN-α and IFN-γ. P2's SV40-fibroblasts showed impaired activation of the ISGF3-complex in response to IFN-α, but normal activation of the GAS-complex in response to IFN-γ (data not shown). Supershift assays of the ISRE- and GAS-binding proteins were performed on SV40-fibroblasts extracts of P2 and her healthy sibling after stimulation with IFN-α and IFN-γ, using antibodies against STAT1, STAT2 and STAT3. In P2 and her healthy sibling, the GAS-binding proteins produced in response to IFN-γ contained STAT1 (GAF-complex). In the healthy sibling, ISRE-binding proteins in response to IFN-α contained both STAT1 and STAT2 (ISGF3-complex), however in P2, no STAT2 protein was detected upon stimulation with IFN-α (data not shown).

Finally, to confirm that the STAT2 defect is complete, *STAT2* cDNA containing either the c.*G1576A* or the c.*C1528T* variant, resulting in the p.Gly522Arg mutation or the premature stop codon p.Arg506X, was overexpressed in a STAT2-deficient cell line (U6A) and *STAT2* expression was measured by Western Blot (refer to the OR for Methods). While *STAT2* expression was absent following overexpression of the mutant allele carrying the p.Arg506X mutation, indicating that the *c.C1528T* variant drives nonsense-mediated decay, normal *STAT2* expression was found following

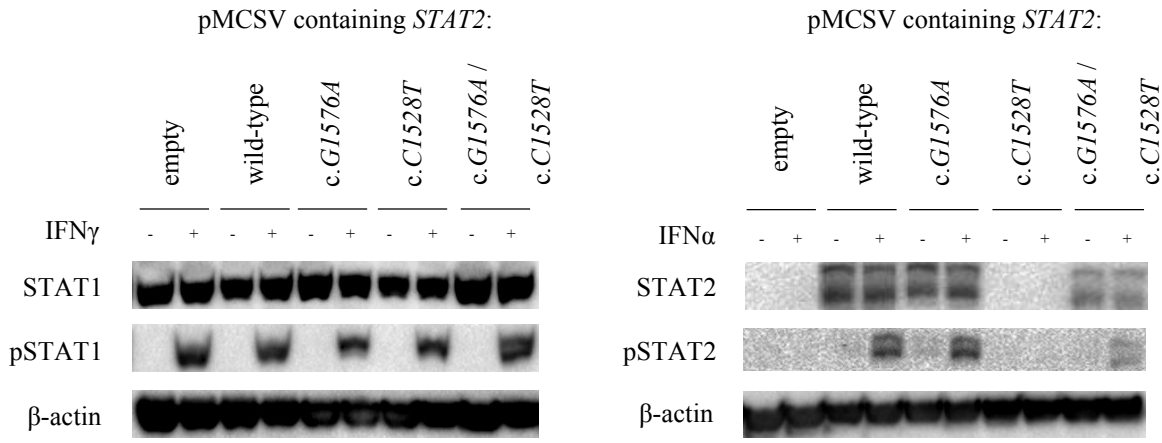


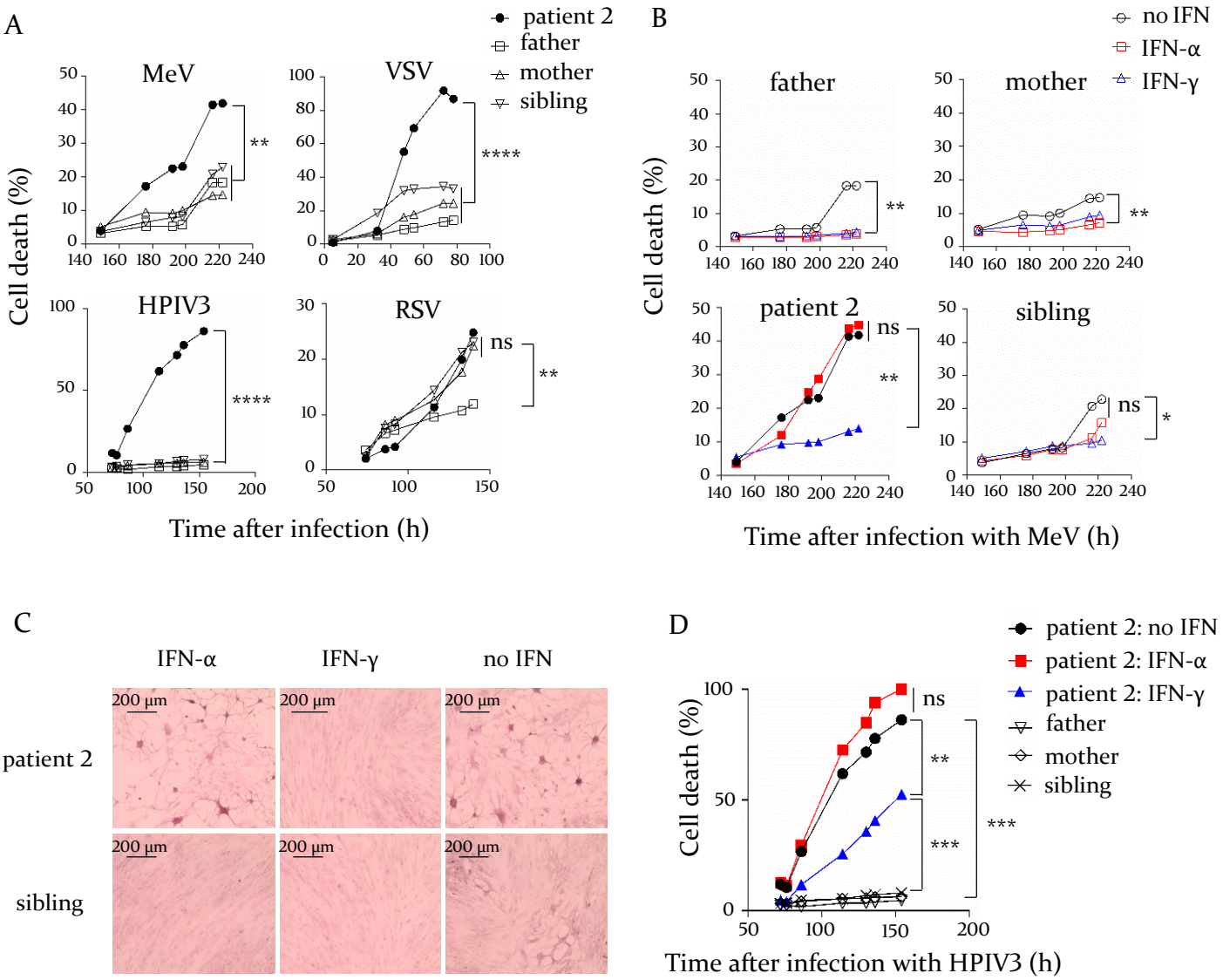
Figure 4**: Overexpression of different *STAT2* variants in a STAT2-deficient cell line (U6A).** U6A cells were transfected with plasmids containing either wild-type *STAT2*, c.*G1576A STAT2,* c.*C1528T STAT2,* both c.*G1576A STAT2* and c.*C1528T STAT2* or plasmid without *STAT2* insert. 72 hours after transfection cells were treated with either IFN-α or IFN-γ at 10^3 Units per ml for 30 min or left untreated. Expression of STAT1, STAT2, phosphorylated STAT1 and phosphorylated STAT2 was verified on whole cell lysates by Western Blot.

overexpression of the mutant allele carrying the p.Gly522Arg mutation (Figure 4). The c.*G1576A* variant occurs in the late exonic position, and Human splicing finder 3.0 [104] predicts that it alters/destroys the wild-type donor site which will probably affect splicing. It is therefore likely that the c.*G1576A* variant is a coding splice site variant, and that aberrant splicing is responsible for the loss of expression of the STAT2 allele carrying this variant. Analysis of *STAT2* transcripts in PBMCs, primary fibroblasts and EVB-transformed B cells of P2 and her mother revealed multiple splicing variants (data not shown). Previous immunoblotting for STAT2 and phosphorylated STAT2 after stimulation with IFN-α demonstrated absence of expression of both full length and truncated protein in P2. Immunoblotting showed reduced expression and phosphorylation in both parents compatible with their carrier status, and absence of truncated or different-length STAT2 protein as well. Given that the antibody used for detecting STAT2 is directed against an epitope located at the N-terminus (amino acids 1-178), and that this nucleotide regions is present in the identified *STAT2* transcripts, it is highly probable that an aberrant or truncated STAT2 protein would have been

detected by Western Blot if it was translated in PBMCs or primary fibroblasts of P2 and her mother. Together with the findings of failure to upregulate ISGs and the impaired activation of the ISGF3-complex in response to IFN-α, we conclude that the examined cell types of P2 are in fact STAT2-null.

Increased susceptibility of patient's primary fibroblasts to in vitro viral infections

Next, we tested P2's susceptibility to viral infections *in vitro* by measuring cell death in fibroblast cultures of all family members infected with a panel of negative-strand RNA viruses (Measles virus (MeV), Vesicular stomatitis virus (VSV), Human parainfluenza virus type 3 (HPIV3) and Respiratory syncytial virus (RSV)) (refer to the OR for Methods). We observed increased cell death in P2 compared to healthy family



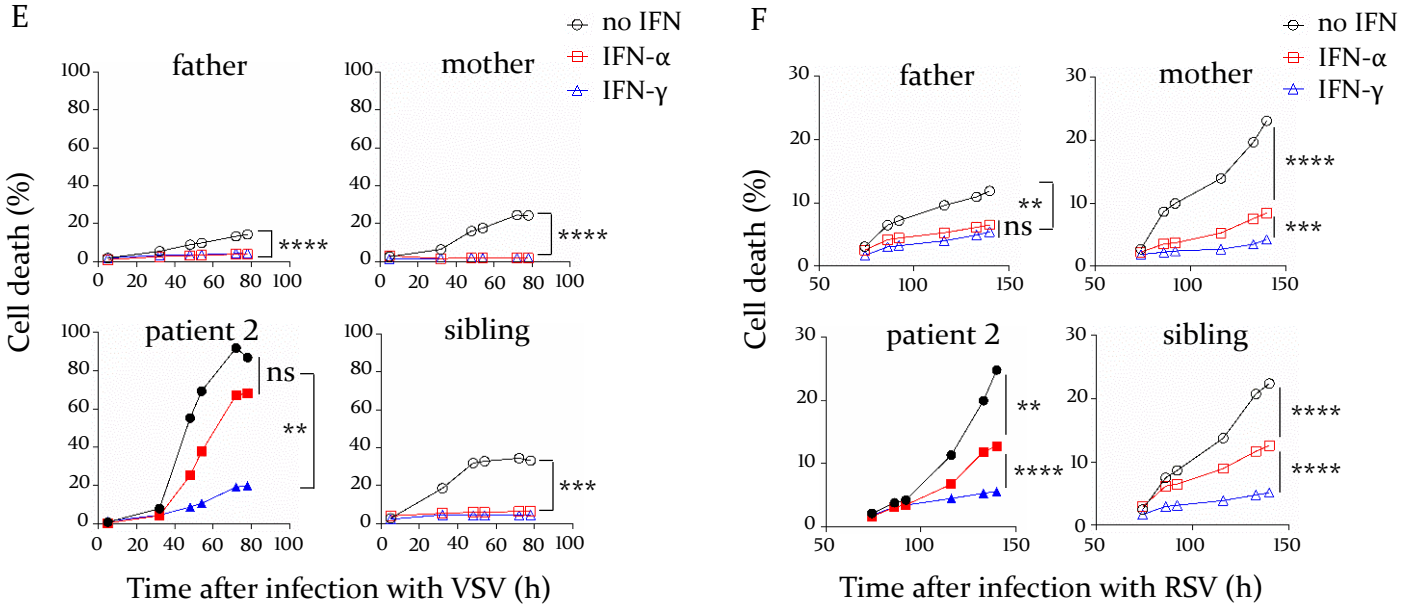


Figure 5**: Increased susceptibility of patient fibroblasts to *in vitro* infection with negative-strand RNA viruses.** A) Fibroblasts of all family members were infected with *Measles virus* (MeV), *Vesicular stomatitis virus* (VSV), *Human parainfluenza virus type 3* (HPIV3) and *Respiratory syncytial virus* (RSV). Cell death over time was measured with Celltox Green Cytotoxicity Assay. B) Fibroblasts of all family members were pre-treated with IFN-α and IFN-γ at 10^3 U/ml for 24h and subsequently infected with MeV. Cell death over time was measured with Celltox Green Cytotoxicity Assay. C) Fibroblasts of patient 2 and her healthy sibling were pre-treated with IFN-α and IFN-γ at 10^3 U/ml for 24h and subsequently infected with MeV. Cells were fixed at day 7 post-infection and visualized by H&E staining. D, E and F) Fibroblasts of all family members were pre-treated with IFN-α and IFN-γ at 10^3 U/ml for 24h and subsequently infected with HPIV3, VSV and RSV respectively. Cell death over time was measured with Celltox Green Cytotoxicity Assay. P-values were calculated for comparison of slopes generated by linear regression. ****, $p < 0{,}0001$; ***, $0{,}0001 < p < 0{,}001$; **, $0{,}001 < p < 0{,}01$; *, $0{,}01 < P < 0{,}05$; ns, not significant.

members after infection with MeV, VSV and HPIV3, but not RSV (Figure 5A). Pretreatment with exogenous IFN-α and IFN-γ protected the fibroblasts of healthy family members from *in vitro* infection with MeV, VSV and HPIV3 while for P2 pretreatment with IFN-γ was protective but IFN-α had no effect (Figure 5B, 5C, 5D and 5E). These results are compatible with disease caused by unresponsiveness to IFN-α, without altering the capacity to signal via STAT1 in response to IFN-γ. Pretreatment with exogenous IFN-γ improved cell survival in all family members during *in vitro* RSV infection, but pre-treatment with IFN-α only had a partial effect in all family members except the father. Strikingly, a partial effect of IFN-α pretreatment was observed in patient fibroblasts as well. Additionally, no significant difference was detected in the response to IFN-α of patient fibroblasts compared to fibroblasts of her other family members (Figure 5F).

To unambiguously establish a causal relationship between the *STAT2* mutations and the cellular/clinical defect in P2, a rescue of the cellular anomaly (viral susceptibility and unresponsiveness to IFN-α) was performed by introduction of wild-type STAT2 cDNA in SV40-transformed fibroblasts of P2. Two strategies were followed to achieve stable expression of wild-type STAT2: 1) development of a Murine stem cell virus

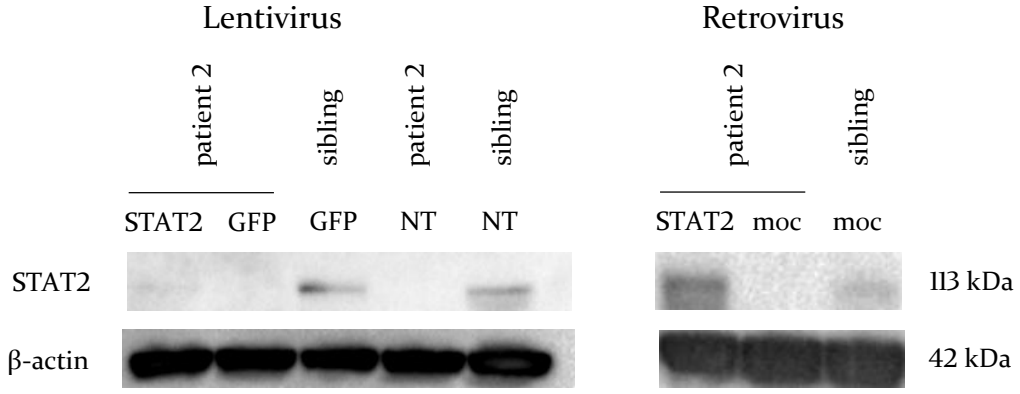


Figure 6**: STAT2 expression in SV40-transformed fibroblasts of patient 2 and her healthy sibling after transduction with lentivirus and retrovirus.** After transduction STAT2 expression was verified on whole cell lysate of SV40-transformed fibroblasts by Western Blot. NT, non-transduced; mock,,retroviral vector without STAT2 insert.

(MSCV) retroviral vector encoding wild-type STAT2 (refer to the OR for Methods), and 2) purchase of a lentiviral vector encoding wild-type STAT2 (Amsbio). Transduction was performed on SV40-transformed fibroblasts given the higher transduction efficiency in this cell type compared to primary fibroblasts. Transduction with the lentivirus resulted in very low STAT2 expression in P2's SV40-transformed fibroblasts. In contrast, transduction with the retrovirus led to high expression of wild-type STAT2 in SV40-transformed fibroblasts of P2 (Figure 6).

While repeating the viral experiments on SV40-transformed fibroblasts of all family members and a STAT1-deficient patient and on the STAT2-deficient cell line U6A, a different response of SV40-transformed fibroblasts of the family members to *in vitro* viral infection was detected compared to the response of their primary fibroblasts (registered by daily monitoring and observation by bright-light microscope). Most strikingly, very little cell death was observed in SV40-transformed fibroblasts of the mother infected with VSV, MeV and RSV: the cells appeared to have become resistant to viral infection and the course of cell death mimicked that of cells pretreated with IFN-α or IFN-γ. In contrast, without pretreatment the SV40-transformed fibroblasts of the father seemed to be almost as susceptible to these viruses as the SV40-fibroblasts of the patient. These findings correlated with the ISG upregulation observed by RT-qPCR in SV40-transformed fibroblasts of the mother and very low ISG expression in SV40-transformed fibroblasts of the father in untreated condition. Additionally, the STAT2-deficient cell line U6A did not support the conditions of the viral assay, namely the low percentage of fetal bovine serum and prolonged culture, high levels of cell death were observed in the first two days of the experiment in all experimental conditions. Therefore, it was concluded that SV40-transformed fibroblasts were not ideal for the viral assays and that STAT2-deficient cell line U6A could not be used as a control, but that the viral assays and the rescue experiment should be performed on primary fibroblasts.

In order to achieve stable and efficient expression of wild-type STAT2 in primary fibroblasts, a lentiviral vector suitable for transduction of primary fibroblasts and encoding both wild-type STAT2 and GFP linked by the 2A peptide from the foot-and-mouth disease virus (FDMV) (F2A) or only GFP (mock vector) will be developed.

Primary fibroblasts of all family members will be transduced so that they can serve as controls for P2's fibroblasts. Primary fibroblast cultures with stable expression of wild-type STAT2 will be generated by sorting of efficiently transduced fibroblasts co-expressing wild-type STAT2 and GFP, and by culturing the cells under selective pressure. These experiments and the finalisation of this project are ongoing.

DISCUSSION

STAT2-deficiency has been proposed as the cause of unusually severe viral illness in infancy [1]. Here we report on a third, unrelated family with STAT2 deficiency in two siblings who suffered from frequent and severe viral infections. Deficiency in these patients appears complete, at least in the few tested cell types, based on protein expression, absent STAT2 phosphorylation, absent binding of ISGF3 to ISRE, and absence of induction of ISGs following IFN-α stimulation. Physical and mental development of both siblings was normal, except for a tendency for dental caries and erosion in both siblings and to a lesser extent in their parents. Enamel defects, although more severe, have been described in STAT3 deficiency and STAT1 gain-of-function mutations [7,8], however it is unclear at this moment whether this dental defect can be attributed to STAT2 deficiency.

Increased susceptibility to vaccine-strain measles with complicated measles infection following routine immunization has been documented in both families, and may be a warning sign for STAT2 deficiency. This susceptibility highlights the importance of type I IFN signalling in the initial immune response against measles infections [9]. 1-3 patients per 1000 measles cases develop primary measles encephalitis (PME), and 4-11 patients per 100,000 measles cases develop subacute sclerosing panencephalitis (SSPE), which is caused by persistent infection of the brain by an aberrant measles virus [9]. Whether STAT2 deficiency or mildly damaging genetic variants in *STAT2* underlie cases of PME and/or SSPE remains to be elucidated.

Hambleton *et al*. reported that in the first described family the majority of childhood viral illnesses were remarkably mild (for example, unremarkable *Varicella zoster* infection and *Herpes simplex virus* (HSV) primo-infection). Although some childhood viral illnesses such as RSV bronchiolitis had a relatively uneventful course in P2, she

did, however, suffer from severe *Varicella* and *Enterovirus* infections requiring multiple hospital admissions. The recurrent viral respiratory infections led to chronic inflammatory post-viral lung disease in P2. She also suffered from persistent warts and *Mollusca contagiosa*. In contrast to the STAT2-deficient patients described by Hambleton et al., P2 had a severe course of primary EBV infection with delayed EBV suppression in peripheral blood and CSF, in line with the important role of type I IFN signalling in the initial immune response against EBV [10]. Together, the clinical presentation in this third family seems to be more severe than the one described by Hambleton *et al*. Similar findings have been reported in other innate immune disorders such as TLR3-deficiency, where HSV-1 primo-infection manifestations can range from asymptomatic to life-threatening encephalitis [11]. STAT2 deficiency may display incomplete penetrance for several viral infections, except perhaps for infection caused by the live attenuated measles vaccine.

Hambleton *et al*. reported a decrease in frequency and severity of viral infections with increasing age [1], similar to the clinical evolution in innate immune disorders affecting TLR signalling pathways, where individuals who survive beyond childhood largely outgrow their susceptibility to invasive bacterial infections [12]. They hypothesized that this effect is due to the development of adaptive immunity. We observed a similar effect in P1 and P2, however, after a relatively infection-free period, P1 suddenly died at age 7 years of an infection with an unidentified pathogen. This demonstrates that STAT2-deficient patients may still be at risk for overwhelming viral illness at a later age, when confronted with a virus to which they are particularly susceptible.

The impaired response to IFN-α and the severe and potentially life-threatening viral diseases in STAT2-deficient patients are reminiscent of the phenotype of patients with autosomal recessive STAT1 deficiency, who additionally suffer from severe infections with weakly virulent mycobacteria and other intracellular bacteria [13]. Their viral phenotype however seems more severe and broader than that of STAT2 deficiency. Hambleton et al. interpreted the apparently normal resistance of STAT2-deficient patients to several viruses as evidence that type I IFN signalling might be redundant in humans. Consistent with the more severe phenotype of STAT1-deficient patients, we favor the alternative hypothesis that there are STAT2-independent cellular responses

to type I IFNs. Similar, albeit less severe, presentation has been observed in patients with autosomal recessive partial STAT1 deficiency [14]. Comprehensive analysis of STAT1-deficient cells strongly suggests that the corresponding patients' viral susceptibility is due to an impaired IFN-α/β response, whereas their susceptibility to intracellular bacteria is due to defective IFN-γ signalling [15].

Finally, we observed a good response to high doses of IVIG in P2 during infectious episodes associated with severe immune dysregulation. This response may in part be due to the anti-inflammatory effect of high doses of IVIG [16], however, it is possible that passive immunization may also play a role in controlling the ongoing viral infections. Therefore it could be argued that IgG replacement therapy might be beneficial for STAT2-deficient patients during childhood, until their adaptive immune system has sufficiently developed, as shown in patients with TIR deficiency [11]. However, the history of P1 has demonstrated that patients may still be at risk for overwhelming viral illness beyond early childhood. Based on our limited experience, we recommend monthly IVIG substitution to prevent infections, and high dose IVIG treatment in the course of severe (viral) infectious episodes with signs of emerging coagulopathy and/or immune dysregulation.

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Online repository

METHODS

The study was performed in accordance with the modified version of the Helsinki declaration. The study was approved by the Ethics Committee of UZ Leuven. Written informed consent was obtained prior to DNA isolation from blood of all family members and from bone marrow of the deceased patient.

Genetic analysis

Whole-exome sequencing. We performed whole-exome sequencing on the surviving patient and on the unaffected parents and younger sibling. Genomic DNA samples for whole-exome sequencing were prepared from heparinized peripheral blood using the QIAamp DNA Blood Midi Kit (QIAGEN, Hilden, Germany). Exome sequence libraries were prepared using a SeqCap EZ Human Exome Library v3.0 kit (Roche NimbleGen, Madison, Wisconsin, USA). Paired-end sequencing was performed on the Illumina HiSeq2000 (Genomics Core Facility, University of Leuven, Belgium). BWA software was used to align the sequence reads to the Human Reference Genome Build hg19. GATK Unified Genotyper was used to identify single nucleotide variants and insertions/deletions. ANNOVAR was used for annotation.

Sanger sequencing. A DNA sample of the transplanted patient was obtained from a bone marrow sample using the GenElute Mammalian Genomic DNA Miniprep Kit (Sigma-Aldrich, St-Louis, USA). The regions of interest in exon 17 of STAT2 were sequenced using the primers 5'- GTTCTGCCCTGTGGGACAGATAG -3' and 5'- CTCAATTGCCTGGGCTTCAGTTC -3'. Sanger sequencing was performed on an ABI 3730 XL Genetic Analyzer (Applied Biosystems) at the LGC Genomics Facility in Berlin, Germany. Sequencing data were analyzed using CLC Main Workbench 6.9.1 (CLC Bio, Denmark).

Functional assays

Peripheral blood mononuclear cells (PBMC) were isolated from heparinized blood of the patient, family members and two unrelated controls using lymphocyte separation

medium (LSM, MP Biomedicals) and cultured at 2x 10^6 cells per ml in RPMI medium with 10% FBS and supplemental penicillin and streptomycin. Dermal fibroblasts were obtained from skin biopsies of the patient, family members and two unrelated controls and cultured in DMEM medium with 10% FBS and supplemental penicillin and streptomycin.

Western blot analysis. Dermal fibroblast cultures and PBMC of the patient, family members and two unrelated adult controls were stimulated with IFN-α 1000 U/mL (ThermoFisher Scientific, Waltham, MA) or IFN-γ 1000 U/mL (R&D systems, Mineapolis, MN) for 30 minutes, or left unstimulated. Cytoplasmic and nuclear extracts were prepared using a nuclear extraction kit (Active Motif Corp, Carlsbad, CA) according to the manufacturer's instructions. Cytoplasmic protein lysates (20 µg per sample) were subjected to SDS-PAGE separation on 4-12% Bis-Tris Plus gels (Life Technologies, Carlsbad, CA), transferred to a polyvinylidene difluoride membrane (GE Healthcare, Buckinghamshire, United Kingdom) and immunoblotted with primary antibodies against STAT1 p84/p91 (M-22, sc-592; Santa Cruz Biotechnology, Dallas, TX), STAT2 (22/Stat2, 610187; BD Biosciences, San Jose, CA) and loading control PAN 14-3-3 (K-19, sc-629; Santa Cruz Biotechnology, Dallas, TX). Nuclear protein lysates were treated in a similar way but were immunoblotted with primary antibodies against phospho-STAT1 (Tyr701, sc-7988; Santa Cruz Biotechnology, Dallas, TX), phospho-STAT2 (Tyr689, AF2890; R&D Systems, Mineapolis, MN) and loading control heterogeneous nuclear ribonucleoprotein I (3H7, sc-73391; Santa Cruz Biotechnology, Dallas, TX). Primary antibodies were detected with horseradish peroxidase–conjugated secondary antibody (sc-2317 and sc-2005, Santa Cruz Biotechnology, Dallas, TX). All Western blot images were captured and quantified with a ChemiDoc MP imager and Image Lab software (Bio-Rad Laboratories, Hercules, CA) after adding Pierce ECL Western blotting substrate (Thermo Scientific, Waltham, MA). STAT1 and STAT2 expression was normalized to the respective value for PAN 14-3-3 expression, phosphorylated STAT1 and phosphorylated STAT2 expression was normalized to the respective value for heterogeneous nuclear ribonucleoprotein I expression.

Viral Assays. Dermal fibroblasts of the patient and the family members were seeded in 96-well plates at 2x 104 cells per well in 200 µL DMEM medium with 2% FBS and

supplemental penicillin and streptomycin, and were stimulated with IFN-α 1000 U/mL (Thermo Scientific, Waltham, MA) or IFN-γ 1000 U/mL (R&D systems, Mineapolis, MN) for 24 hours, or left unstimulated. Cells were infected with *measles virus* (strain Edmonston-Zagreb, MOI 0.03), *vesicular stomatitis virus* (strain Indiana, MOI 0.005), *human parainfluenza virus type 3* (strain C243, MOI 0.05) or with *respiratory syncytial virus* (strain Long, MOI 0.003). Cell death post infection was measured for 72h using CellTox™ Green Cytotoxicity Assay (Promega, Madison, WI) according to the manufacturer's instructions. Measurements were started at 144 hours post infection for *measles virus*, 6 hours post infection for *vesicular stomatitis virus* and 72 hours post infection for *human parainfluenza virus type 3* and *respiratory syncytial virus*. Experiments were performed in technical replicates and were repeated three times. A representative experiment was shown in Figure 2.

Statistical analysis of viral assays. Linear regression was performed: for each virus, individual and treatment condition, the slope of cell death in function of time was calculated. For each virus, the slope calculated for healthy individuals without IFN pretreatment was compared to that of the patient in this condition, or within each individual the slopes calculated for IFN pretreatment were compared with the slope calculated for the untreated condition.

Real-time quantitative polymerase chain reaction (RT-qPCR). Total RNA was isolated from SV40-transformed fibroblasts and primary fibroblasts with TrizolReagent (Invitrogen) by following the manufacturer's instructions. Complementary DNA (cDNA) was synthesized by reverse transcription using the SuperScript Vilo cDNA synthesis kit (Life Technologies). The cDNA was analyzed by real-time quantitative PCR. An appropriate amount of the cDNA was mixed with TaqMan Gene Expression Master Mix (Life Technologies) supplemented with gene-specific Taqman probes: for *MX1* (Hs00895608-m1, Life technologies), for *OAS1*, (Hs00973635-m1, Life technologies), for *ISG15* (Hs00192713-m1, Life technologies), for *ACTB*, (Hs01060665-g1, Life technologies), and for *GUSB* (Hs00939627-m1, Life technologies). Real-time quantitative PCR analysis was performed on a StepOnePlus realtime PCR system (ABI). The resultant PCR products were analysed with ABI 7500 software (Applied Biosystems). Gene expression was analysed with the 2–ΔΔCt method [10], and all

quantifications were normalized to the average of the level of *ACTB* and *GUSB*. Experiments were performed in triplicate and repeated twice on primary fibroblasts. Statistical analysis was performed by regular two-way Anova and Tukey multiple comparisons tests are used to correct for multiple comparisons.

Electrophoretic mobility shift assay (EMSA). EMSA was performed as described before by Stéphanie Boisson-Dupuis[134]. SV40-transformed fibroblasts were stimulated with 10^5 IU IFN-γ per ml or 10^5 IU IFN-α per ml for 30 minutes. 10 µg of nuclear extracts were used for assessment of GAS-binding proteins, and 20 µg of nuclear extracts for ISRE-binding proteins. For supershift assays, antibodies against STAT1 (sc345X), STAT2 (sc476X), STAT3 (sc7179X) and p48/IRF9 (sc496X) (Santa Cruz) were used, and rabbit IgG (Sigma) as an isotypic control.

Overexpression experiments. Full-length ORF of wild-type STAT2 was amplified using PrimeSTAR GXL DNA Polymerase (Takara-Bio) (forward primer: 5'-AAAAAACTCGAGATGGCGCAGTGGGAAAT GCTG-3', reverse primer: 5'-AAAAAAGAATTCCTAGAAGTCAGAAGGCAT-3') and cloned into the pMCSV puro plasmid backbone (Clontech) between the XhoI/EcoRI restriction sites under a constitutively active LTR-promotor. To generate the p.Gly522Arg and p.Arg506X mutations in STAT2, site-directed mutagenesis was undertaken using the Q5 Site-Directed Mutagenesis Kit (New England Biolabs) (forward primer p.Gly522Arg: 5'-TCAATTACAAaGCTTCCGGAAG-3', reverse primer p.Gly522Arg: 5'-GGAGGATTCCTGTCAATG-3', forward primer p.Arg506X: 5'-CTATGTTGGCtGAGGCCTCAA-3', reverse primer p.Arg506X: 5'-GAGGAGAACTGCCAACTG-3'). 10^6 STAT2-deficient cells (U6A) were transfected with 5 µg of plasmid DNA containing either wild-type STAT2, p.Gly522Arg STAT2, p.Arg506X STAT2 or a mock vector, using lipofectamine 3000 (Thermo Fischer Scientific). 72 hours after transfection cells were stimulated with IFN-α 10^3 IU/ml and IFN-γ 10^3 IU/ml for 30 minutes, subsequently harvested and protein extracts were prepared and immunoblotted for STAT2 and phosphorylated STAT2 as previously described.

Transduction of SV40-fibroblasts with wild-type STAT2. Full-length ORF of wild-type STAT2 was cloned into the pMCSV puro plasmid backbone (Clontech) as previously described. This pMSCV puro plasmid contains a puromycin resistance gene as a selectable marker. Retroviruses were produced and viral stocks were concentrated using Retro-X concentrator (Clontech) according to the manufacturer's instructions. SV40-fibroblasts of the patient were transduced using Polybrene (8μg/ml) and an inoculation spin (1,200 x g for 60 min at 32°C). After 48 hours transduced cells were selected for puromycin resistance (0.25 μg/ml) and left to proliferate under selective pressure (puromycin 0.125 μg/ml). STAT2 expression was verified by Western blot as previously described. Viral experiments were repeated on the transduced cells as previously described, after puromycin treatment was stopped for 7 days.

TABLES

Table E1: Lymphocyte subsets and Ig measurements in patient 2.

Leucocyte subset or Ig	2 years	5 years	10 years
Neutrophils (kU/µL)	6,4 (1,5 – 8,5)	2,6 (1,5 – 8,5)	4,7 (1,8 – 8,0)
Lymphocytes (kU/µL)	4,3 (2,7 – 11,9)	2,9 (1,7 – 6,9)	2,5 (1,5 – 6,5)
T cells (kU/µL)	2,2 (1,4 – 8,0)	1,7 (0,9 – 4,5)	1,4 (0,7 – 4,2)
$CD4^+$ T cells (kU/µL)	1,4 (0,9 – 5,5)	0,89 (0,5 – 2,4)	0,82 (0,5 – 2,4)
$CD8^+$ T cells (kU/µL)	0,74 (0,4 – 2,3)	0,64 (0,3 – 1,6)	0,51 (0,3 – 1,6)
NK cells (kU/µL)	0,15 (0,1 – 1,4)	0,68 (0,1 – 1,0)	0,59 (0,1 – 1,0)
B cells (kU/µL)	1,05 (0,6 – 3,1)	0,51 (0,2 – 2,1)	0,65 (0,2 – 2,1)
Switched memory B cells	NA	13.4 %	13.5%
IgG (g/L)	16.2 (3.02 – 9.85)	11.7 (4.78 – 11.29)	11.8 (5.30 – 13.06)
IgA (g/L)	0.78 (0.13 – 1.08)	0.89 (0.35 – 1.90)	1.06 (0.60 – 2.70)
IgM (g/L)	1.32 (0.26 – 1.60)	0.54 (0.34 – 1.34)	0.49 (0.43 – 1.73)
IgE (kU/L)	47 (<91)	1177 (<224)	1127 (<331)
Measles Ab	protective	protective	NA
Poliomyelitis Ab	protective	NA	NA
EBNA	absent	protective	NA
Hepatitis B Ab	protective	absent	NA

NK cells, natural killer cells; EBNA, *Epstein-Barr virus* nuclear antigen; NA, not available.

Table E2: EBV measurement by PCR in patient 2.

Age (years)	**2,5**	**3**	**4**	**4,5**	**5,5**	**10**
EBV (log copies/ml)	4.73	2.99	4.25	3.07	4.33 (blood) 2.71 (CSF)	undetectable

EBV, Epstein-Barr virus; CSF, cerebrospinal fluid; PCR, polymerase chain reaction.

DISCUSSION

1. A *de novo* heterozygous *IFIH1* mutation in a 16-year-old girl with severe early-onset and refractory systemic lupus erythematosus, IgA-deficiency and mild lower limb spasticity without neuroradiological manifestations

1.1 Modifying factors in diseases associated with IFIH1 gain-of-function mutations

Gain-of-function mutations in *IFIH1* have been identified in a spectrum of disorders associated with elevated levels of type I IFN, such as AGS [135], HSP [42] and Singleton-Merten syndrome (SMS), a rare autosomal-dominant disorder characterized by early and extreme aortic and valvular calcification, dental anomalies (early-onset periodontitis and root resorption), osteopenia, and acro-osteolysis [136]. The term 'type I interferonopathies' has been used to describe this novel group of inborn errors of immunity associated with upregulation of type I IFN [137]. Genetic defects in genes known to be involved in type I interferonopathies are often associated with a wide range of clinical manifestations. This is clearly the case for mutations in *IFIH1*, but is for example also described in *TREX1* deficiency which can be associated with AGS and SLE, but also with familial chilblain lupus and with retinal vasculopathy with cerebral leukodystrophy [138]. Interestingly, *IFIH1* gain-of-function mutations are also found in healthy family members of patients. However, all patients as well as asymptomatic family members carrying the *IFIH1* mutation had a profound IFN signature [135]. Therefore it seems possible that *IFIH1* gain-of-function mutations can function as a major predisposing factor for the development of type I interferonopathies, but that they are insufficient to drive disease on their own and that other genetic, environmental, or stochastic modifying factors leading to for example a break in tolerance play a role in disease development.

The possibility of sIgAD acting as a modifying factor in the development of SLE in the patient described in this work has been discussed in detail in the publication. This effect could be partially environmental (increased exposure to viral antigens) and partially genetic (loss of the protective/inhibitory role of IgA against autoimmunity). Identifying other genetic modifying factors by for example GWAS or by assessing the

burden of known SLE or sIgAD risk alleles identified by GWAS, is not possible in a single patient. It would be interesting though to perform this type of genetic analysis on a cohort of individuals with *IFIH1* gain-of-function mutations, to gain insight into the interplay between a Mendelian mutation and common risk variants in *IFIH1*-associated type I interferonopathies. However this would require a sufficiently large group of patients to provide the necessary power to answer this question.

1.2 Treatment of type I interferonopathies

Immunophenotyping of the patient described in this work led to the identification of an immunological signature, i.e. both cellular and humoral disturbances, that was previously found in patients with active SLE. The finding of persistent disease activity linked to high serum levels of IFN-α as well as the identification of the genetic defect and cellular pathways responsible, suggests the IFN-driven nature of the disease and provides a rationale for trying targeted inhibition of IFIH1 or the type I IFN-pathway to control disease in this patient. Targeted inhibition of the type I IFN-pathway may be beneficial for all individuals with (*IFIH1*-associated) type I interferonopathies, not only for controlling the immunological disease manifestations but perhaps also for preventing further disease progression, for example further neurological deterioration in AGS. Examining the clinical course in AGS has shown that a therapeutic window of opportunity exists, and that assessment of type I interferon activity might serve as a useful biomarker [139]. However, the assumption here would be that type I IFN is the (sole or most important) driver of neurological deterioration in AGS, which remains a hypothesis at this time.

As mentioned briefly in the article, IFIH1 senses long double-stranded RNA (dsRNA) and mediates an anti-viral response by activating type I interferon signalling [140]. After binding with (viral) dsRNA, IFIH1 interacts with the adaptor molecule mitochondrial antiviral signalling protein (MAVS) which leads to the production of type I IFN through recruitment of IRF3 and production of pro-inflammatory cytokines through activation of NF-κB. Subsequently type I IFN acts on cells expressing IFNAR by activating the JAK-STAT pathway downstream of IFNAR and inducing the expression of hundreds of IFN-stimulated genes (ISGs) (Figure 4). The resulting gene

products have a broad effect on multiple cell types: 1) they initiate an intracellular antiviral program in all cells expressing IFNAR to limit viral replication and further spreading of infectious agents, 2) they enhance antigen presentation and the production of cytokines and chemokines by innate immune cells and 3) they activate adaptive immunity, for example by augmenting antibody production by B cells [141]. Clinical trials with several molecules that target IFN-α or its receptor, or the signalling

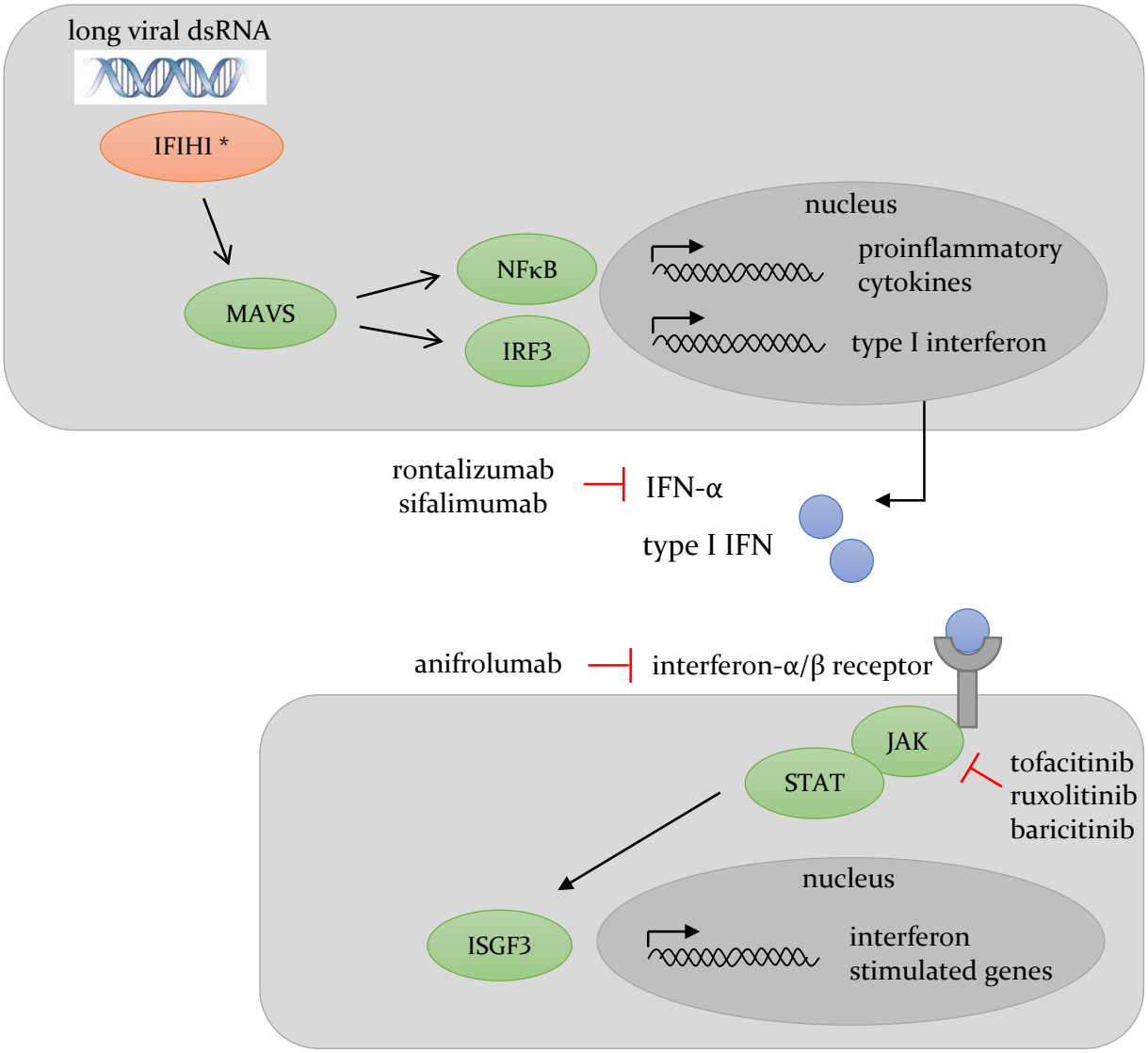

Figure 4: **IFIH1-mediated type I IFN signaling and potential treatment strategies.** dsRNA, double-stranded RNA; IFIH1, interferon induced with helicase C domain 1; IRF3, interferon regulatory factor 3; JAK, Janus kinase; MAVS, mitochondrial antivirus signaling protein; NF-κB, nuclear factor kappa B; STAT, signal transducer and activator of transcription; ISGF3, interferon-stimulated gene factor 3.

cascade downstream of IFNAR (JAK-inhibitors), are either underway or have demonstrated their (lack of) effectiveness.

Several biologicals that target IFN-α, e.g. sifalimumab or rontalizumab, have been developed and tested in phase II clinical trials [142, 143]. Although these drugs had acceptable safety profiles and treatment led to a dose-dependent reduction of the IFN signature in peripheral blood of SLE patients, the clinical effect was disappointing, with no significant reduction in disease activity compared to placebo in patients with moderate to severely active extrarenal SLE. Hence, it is possible that direct targeting of IFN-α might not be successful in controlling SLE. An alternative approach would be to block IFNAR, which is expected to block the activity of all type I IFN types, including IFN-β. Therefore, efficacy but also toxicity might be higher compared to biologicals directly targeting IFN-α. A phase II trial of anifrolumab, a monoclonal antibody against subunit 1 of IFNAR that inhibits the activity of all type I IFNs, demonstrated a significant reduction of disease activity compared to placebo in adult patients with moderate to severe SLE [144]. Occurrence of severe adverse events was similar in placebo or anifrolumab treated patients apart from a dose-dependent increase in *Herpes zoster* as well as (mostly unconfirmed) influenza infections. Subsequently, an anifrolumab phase III program was started [145]. Overall, IFNAR blockade could be a promising strategy for the treatment of moderate to severe SLE, and could be beneficial for *IFIH1*-associated type I interferonopathies.

A third possibility would be to block the signalling cascade downstream of IFNAR by using JAK-inhibitors. At the moment no evidence is available on the effectiveness of JAK-inhibitors in patients with SLE, but improvement of autoimmunity and nephritis was observed in lupus prone mice treated with tofacitinib, a JAK1/3-inhibitor already used for treatment of rheumatoid arthritis [146]. Also, treatment with ruxolitinib, a JAK1/2 inhibitor, lead to significant clinical improvement in three patients with aleopecia areata [147], and in a patient with *JAK2 V617F*-positive post–polycythemia vera myelofibrosis and dermatomyositis [148]. A phase I trial testing the safety of tofacitinib in SLE is currently being undertaken. Additionally, treatment with the JAK1/2-inhibitor baricitinib is also being tried [149] in other type I interferonopathies such as chronic atypical neutrophilic dermatosis with lipodystrophy and elevated

temperature (CANDLE) [150] or stimulator of interferon genes (STING)-associated vasculopathy with onset in infancy (SAVI) [151]. The disease mechanism in SAVI, an autoinflammatory disorder due to gain-of-function mutations in STING, is similar to that of *IFIH1*-associated type I interferonopathies. In SAVI constitutively activated STING leads to increased production of IFN-β which binds to IFNAR, activates the JAK-STAT pathway and leads to the induction of ISGs. *In vitro* studies have already demonstrated a reduction of the constitutive up-regulation of phosphorylated STAT1 in SAVI patients' lymphocytes by JAK inhibitors [151] and a preliminary study showed significant clinical improvement of two SAVI patients treated with ruxolitinib [152]. If treatment of SAVI with JAK-inhibitors proves to be successful in larger trials, this could also be the case in patients with *IFIH1*-associated type I interferonopathies.

2. Recessively inherited homozygous *CECR1* mutations in a 9-year-old boy with Castleman's-like disease and in two siblings with combined immunodeficiency, lymphoproliferation, autoimmunity and vasculopathy

2.1 Clinical and immunophenotypical heterogeneity in ADA2 deficiency

Recessively inherited loss-of-function mutations in *CECR1*, leading to ADA2 deficiency, were identified in a patient with Castleman's-like disease and in two siblings with CID, lymphoproliferation, autoimmunity and vasculopathy. During the work-up of these single family cases, two other research groups identified disease-causing mutations in *CECR1* in two cohorts of patients. In one cohort patients presented with polyarteritis nodosa (PAN) [70], whereas in the other cohort they presented with systemic inflammation, systemic vasculopathy in the form of early-onset recurrent stroke, livedoid rash or vasculitis, hepatosplenomegaly, hypogammaglobulinemia and lymphopenia [69]. Additionally, mutations in *CECR1* were also identified in a family with Sneddon's syndrome, an autoinflammatory disorder characterized by intermittent fevers, livedoid rash and leg ulcerations [153]. Several affected family members developed early-adulthood stroke as well. These findings demonstrate the important clinical heterogeneity in ADA2 deficiency.

All patient cases published so far presented with some sort of systemic inflammation. In both CID siblings however the inflammation appeared to be subclinical, without

recurrent fevers or increased acute-phase reactants. Only through the use of assays not available in standard clinical practice such as serum IL-6 measurement by Elisa, did the important systemic inflammation become apparent in these patients. Another important feature of ADA2 deficiency, the vasculitis/vasculopathy and inflammatory skin lesions do not always seem to be overtly present either, at least not early in the disease presentation. The patient with Castleman's disease and the transplanted sibling examined in this work never developed livedoid rash or inflammatory skin lesions, however the younger sibling recently developed a livedoid rash at the age of 5 years. Over a follow-up period of seven years up until now the patient with Castleman's-like disease has not developed any signs of vasculopathy. At the time of discovery of the genetic defect vasculopathy was not an overt manifestation in the CID siblings either, however subclinical vasculitis was probably already present. The older brother developed a haemorrhagic stroke during the course of the HSCT, which was thought to be a consequence of a HSCT induction-related cytokine storm and the severe thrombocytopenia at that time. His younger brother however has developed two ischemic strokes over the past year (after publication in Journal of Allergy and Clinical Immunology 2014), as well as a relapse in inflammatory bowel disease. Later disease or stroke onset has been described in other patients as well [70, 153], so careful follow-up of all ADA2-deficient patients is mandatory. Still, the findings in this work demonstrate that ADA2 deficiency should not be excluded based on the absence of overt inflammation and/or clinical vasculitis signs.

Lymphoproliferation, i.e. hepatosplenomegaly and generalized lymphadenopathies, was a prominent feature in all three patients. Concerning the immune phenotype, both CID siblings presented with hypogammaglobulinemia, lymphopenia and a B cell differentiation defect, similar as to what was found by the group of Zhou *et al.* [69]. Although T cell proliferation tests *in vitro* were normal, an *in vivo* defect in T cell activation was observed as well which could account for the patients' increased susceptibility to viral infections (e.g. severe and recurrent cutaneous HSV-1 infection) and their CID-like phenotype. The observed mild T cell defect could also account for the development of autoimmunity (auto-antibodies against erythrocytes and thrombocytes) in these patients, which is often associated with CID or CVID. Results

of extensive *in vivo* immunoprofiling have not been published by the other research groups, but *in vitro* tests of T cell activation were normal as well [69] and the presence and/or absence of autoantibodies is not mentioned [69, 70, 153]. In contrast with this CID-like phenotype, the patient with Castleman's-like disease presented with polyclonal hypergammaglobulinemia, a feature consistent with hyperactivation of B cells. Additionally, in this patient all leucocyte subsets were within normal range compared to healthy age-matched controls.

These clinical and immunophenotypical differences cannot be explained solely by the *CECR1* genotype. The p.Arg169Gln mutation in the CID siblings was previously observed in hemizygous and homozygous form [69, 70] and the homozygous p.Gly47Arg mutation in the Castleman's patient was found in several patients with polyarteritis nodosa [70]. It is possible that other genetic factors might play a role in the development of for example the immunological phenotype. The influence of (relatively rare) genetic polymorphisms on immune phenotype and for example T cell function have been described previously [111], and therefore differences in genetic background could be responsible for the observed immunophenotypic discrepancies.

Three other paediatric patients with HHV8-negative Castleman's-like disease and their unaffected parents were recruited through a collaboration with Professor Isabelle Kone Paut (France), however, sequencing of the coding regions of *CECR1* in patients and parents did not reveal any recessively inherited mutations. The same result was obtained by the 'National Institute of Health' group that described the first cohort of ADA2-deficient patients and that also did not find *CECR1* variants in their cohort of patients with HHV-8-negative Castleman's disease [154]. This is not surprising since Castleman's disease is reportedly associated with a variety of conditions such as underlying systemic inflammatory diseases, paraneoplastic syndrome mechanisms via ectopic cytokine secretion, and/or a non-HHV-8 virus [55].

A striking difference seen in both the patient with Castleman's-like disease and in the two siblings with CID, autoimmunity and lymphoproliferation; is the presence of an IL-6 signature. This is in stark contrast with the previously described ADA2-deficient cases in which no particular cytokine profile was found in serum [69, 70]. No increase

in TNFα was detected in serum of the three patients described here. In two other ADA2-deficient patients an upregulation of ISGs was found in peripheral blood, together with a marked overexpression of neutrophil-derived genes [155]. It is not clear whether type I IFN might play a role in disease pathogenesis, or whether this IFN signature is secondary to the general inflammation. Treatment with anti–interleukin-6 receptor antibody (tocilizumab) resulted in a rapid, complete, and persistent suppression of all clinical features and laboratory abnormalities in the patient with Castleman's-like disease, compatible with the major role of IL-6 in driving the inflammatory phenotype [48, 49]. However, this brings up the question why the high IL-6 levels are not having a similar effect in the two siblings with a CID-like phenotype. They did present with lymphoproliferation, but did not experience recurrent fevers nor increased acute-phase reactants or polyclonal hyperglobulinemia.

2.2 ADA2 deficiency and IL-6?

As mentioned above, an IL-6 signature was found in serum of all three patients, whereas IL-6 was undetectable in serum of other family members and healthy individuals. Immunohistochemical analysis of a specimen obtained on lymph-node biopsy from the Castleman's-like disease patient confirmed strong expression of IL-6, mild expression of IL-1β and absent expression of TNFα, which together with the clinical presentation prompted his initial diagnosis. IL-6 is a cytokine that can be produced and secreted by many cells including monocytes, T cells, fibroblasts and endothelial cells. It can be a pro-inflammatory or protective/regenerative cytokine, depending on the signalling pathway used (IL-6 trans-signalling via the soluble IL-6R or classic IL-6 signalling via the membrane bound IL-6R respectively) [46]. Activation of the NF-κB pathway and IL-1β are strong stimuli for the production of IL-6 and its levels are increased in many if not all infectious and inflammatory diseases [46].

It remains unclear at this moment how ADA2 deficiency could lead to IL-6 production *in vivo*. Answering this question may be difficult, since no ortholog for *CECR1* exists in commonly used animal models such as mice and rat. However, findings in mice with ADA deficiency (the ortholog of human ADA1) suggest that there might be a link between loss-of-function of adenosine deaminase and IL-6: the accompanying increase

in adenosine levels have been reported to stimulate IL-6 induction through activation of the adenosine A2B receptor [156]. Furthermore, increasing evidence has shown that adenosine can drive monocyte differentiation toward pro-inflammatory monocytes/macrophages and increased IL-6 production through the adenosine A2B receptor in human and mice [157, 158]. Neither adenosine nor deoxyadenosine levels were increased in plasma of the patient with Castleman's disease and of the untransplanted sibling with CID-like disease, which correlates with the normal ADA1 activity found in all individuals and the dominant role of ADA1 in adenosine metabolism in physiological conditions and in the blood stream [159]. However, ADA2 may be important for controlling adenosine levels in sites of inflammation or hypoxia where the adenosine concentration is significantly elevated and the extracellular pH is acidic, or locally in different tissues [159]. One hypothesis therefore is that locally increased adenosine levels due to absence of ADA2 could drive the pro-inflammatory macrophage polarization that was previously observed *in vitro* [69] and lead to local IL-6 production through the adenosine A2B receptor as mentioned above. IL-6 can enhance monocyte recruitment [160], rescue T cells from apoptosis [161], and boost proinflammatory T cell polarization [162], which could promote chronic inflammatory cell infiltrates. Through IL-6 trans signalling it could promote the release of additional IL-6 from T cells, fibroblasts and endothelial cells in a positive autocrine feedback system [46]. Finally, IL-6 could then have systemic effects when it enters the blood stream, whereas adenosine will be immediately metabolized by ADA1 when it does the same.

It was hypothesized that ADA2 deficiency may compromise endothelial integrity in small blood vessels. Substantial endothelial damage as well as endothelial-cell activation has been shown in patient biopsy specimens from the brain and from skin lesions [69]. Furthermore, small hairpin RNA (shRNA) knockdown of ADA2 in myeloid U937 cells led to considerable disruption of cocultured monolayers of human primary dermal microvascular endothelial cells, as did monocytes from ADA2-deficient patients [69]. The role of IL-6 has been demonstrated in endothelial activation and dysfunction, as well as its involvement in several types of vasculitis and in other disorders associated with compromised endothelial integrity such as arterial

aneurysms [162]. Therefore it seems reasonable to assume that the high IL-6 levels in both siblings with CID-like disease were contributing to their vascular disease.

2.3 Treatment of ADA2 deficiency

Despite severe early complications, allogeneic HSCT was successful both in rescuing the immunologic phenotype and in preventing further vascular disease in ADA2 deficiency. During a seven year follow-up, consecutive clinical and biochemical investigations in the transplanted patient have shown no signs of immunologic disorder and no additional strokes. The course of the transplantation and the potential risks have been described in detail in the Journal of allergy and clinical immunology. Briefly, ADA2-deficient patients may be high-risk candidates for HSCT for several reasons. First, the inflammatory response associated with conditioning is superimposed on the inflammatory state intrinsic to ADA2 deficiency, which might negatively affect engraftment. Second, the compromised endothelial integrity observed in patients with ADA2 deficiency could predispose to development of veno-occlusive disease (VOD), a potentially fatal complication of HSCT. Third, this combination of inflammation and endothelial injury might further increase the risk of stroke in the pre-engraftment and early post-engraftment phases as observed in the transplanted patient. Suggested treatment for preventing these complications include VOD prophylaxis with defibrotide, pretreatment with anti–IL-6R mAbs to control IL-6 levels before the start of the HSCT and to damper the cytokine storm peri-HSCT, or treatment with anti–TNFα mAbs peri-HSCT given its usefulness in preventing and treating acute graft-vs-host disease (GvHD). However, given the underlying immunodeficiency, the risk of infection needs to be carefully balanced when using anti–IL-6R and anti–TNFα mAbs.

ADA2 plasma activity was restored after allogeneic HSCT, which is consistent with bone marrow–derived monocytes and macrophages being the main sources of secreted ADA2. However, whether or not this translates into a restoration of ADA2 plasma activity in other tissues, remains unknown. Whether normal ADA2 plasma activity is sufficient to cure the disease, whether ADA2 plays a role in other tissues and what the effect of absent ADA2 tissue activity on long-term prognosis could be, remains unclear

as well. A report on HSCT in a patient with ADA2 deficiency with a 9-year follow-up is promising [163]. It is plausible that the benefit from HSCT to our patient is entirely due to restoration of normal plasma ADA2 levels. If true, future treatment with exogenous ADA2 might provide an alternative therapy for ADA2 deficiency in patients in whom allogeneic HSCT is contraindicated.

In agreement with findings by other research groups [69, 70], treatment with a variety of immunosuppressive medications resulted in poor disease control in both siblings with CID-like disease. In contrast, treatment with tocilizumab resulted in complete and persistent suppression of all clinical features and laboratory abnormalities in the patient with Castleman's-like disease. Four years after disease onset and three years after the start of the tocilizumab treatment the patient has not experienced any vasculitis or vasculopathy manifestations. While a possible explanation for the curative effect of allogeneic HSCT is available, the mechanisms behind achieving disease control through the use of anti-IL-6R mAbs remain unclear. This result suggests at least a partial contribution of IL-6 to disease pathogenesis in ADA2 deficiency in this patient. ADA2 plasma activity however remains absent in the patient. It is possible that controlling the inflammatory syndrome is sufficient to prevent vascular damage, however it has been hypothesized that ADA2 might also exert growth factor-like activity on endothelial cells [69]. What the effect of absent ADA2 plasma activity on long-term prognosis might be, and whether the patient might still be at risk for developing strokes/vasculopathy at a later age, remains unclear. A recent report on clinical manifestations and treatment of 15 patients with ADA2 deficiency mentions that one patient developed a stroke while under treatment with tocilizumab, and treatment was subsequently changed to the anti-TNFα mAb adalimumab [164]. At the moment, no clinical or biochemical signs suggesting persisting disease activity can be detected in the patient with Castleman's-like disease, however, strict follow-up is necessary. Given the apparent complete control of the disease at this time, it does not seem necessary to change treatment from IL-6 blockade to TNFα blockade and it would be unethical and contraindicated to perform a (risky and potentially dangerous) allogeneic HSCT in this patient. However, if the patient were to develop strokes or other signs of vasculopathy, these therapeutic options would have to be reconsidered.

As more ADA2-deficient patients will be identified in the future and longer follow-up of other potential treatments such as TNFα blockade will become available, a more evidence-based decision can be made.

Treatment with tocilizumab led to complete normalisation of the immunological disturbances (systemic inflammation and lymphoproliferation), but also had an important effect on other clinical disease manifestations. The patient also presented with significant growth retardation. In fact, after the development of disease, the patient's growth ceased completely. However, after the start of the tocilizumab treatment an important catch-up growth was observed. His phenotype pre-tocilizumab resembled that of IL-6 transgenic mice, where overexpression of IL-6 during the prepubertal stage results in stunted growth, abnormalities of the insulin-like growth factor I system, defective growth plates, delayed development of ossification centres, uncoupling of osteoblast and osteoclast activity and defective ossification [165]. Similar growth delays were observed in the two siblings with CID-like disease, and catch-up growth was observed in the older sibling post-HSCT. Additional factors such as glucocorticoid treatment may probably have played a role in the older sibling but cannot completely account for this, as the younger sibling only received treatment with IVIG for at least one year. These findings demonstrate the important effect of (systemic) inflammation on growth, and the necessity of treatments aiming at controlling this inflammation.

3. Recessively inherited compound heterozygous STAT2 mutations in two siblings with severe viral illness

3.1 The protective effect and potentially compensating role of IFN-γ during viral infections in STAT2-deficient patients

IFN-γ rescues the viral phenotype of STAT2-deficient fibroblasts in the *in vitro* viral assay, whereas IFN-α does not. This finding however raises the question why STAT2-deficient patients suffer from severe viral disease if IFN-γ can compensate for this cellular anomaly? Alternatively, it suggests that the outcome of the *in vitro* viral assay is not relevant for the clinical disease course in STAT2-deficient patients.

The *in vitro* viral assay is an artificial system, in which a monoculture of primary fibroblasts is infected with several (-) single-stranded (ss)RNA viruses 24 hours after addition of exogenous IFN-α or IFN-γ. Under these circumstances IFN-γ could (partially) protect patient fibroblasts from viral infection whereas IFN-α could not. This protection is pathogen-dependent and might not be complete, as infection with human parainfluenza virus type 3 (HPIV3) still led to considerable cell death in patient fibroblasts despite pretreatment with IFN-γ, in contrast with fibroblasts from other family members who were not susceptible to HPIV3-infection. This experimental set-up obviously does not recapitulate what happens *in vivo* during a viral infection.

After the start of an infection *in vivo,* type I IFN is produced by infected cells in response to the detection of the pathogen [141]. All cells can produce IFN-β with fibroblasts being one of the main producers, whereas IFN-α is mainly produced by hematopoietic cells, particularly pDCs. The first effect of type I IFN is the induction of a cell-intrinsic antiviral state in infected and neighbouring cells to limit further spread of the viral pathogen. Additionally, type I IFN modulates innate immune responses by promoting antigen presentation and NK cell functions, and activates the adaptive immune system, thus promoting the development of high-affinity antigen-specific T and B cell responses and immunological memory [141]. In contrast, type II IFN is produced by activated NK cells and T lymphocytes, in response to IL-12 and IL-18 produced by antigen presenting cells (APCs) detecting viral infection [166]. There is an important overlap with the effect of type I IFN, although compared to type I IFN, IFN-γ has more pro-inflammatory effects given its role in macrophage activation [166]. However, IFN-γ also becomes important later in the antiviral response by coordinating the innate and adaptive immune response and establishing an antiviral state for longer term control [166].

In the *in vitro* viral assay on fibroblast monocultures the response to both IFN-α and IFN-γ pretreatment is limited to the cell-intrinsic effects, and the assay clearly shows that primary fibroblasts of the patient have an increased viral susceptibility and fail to mount this initial cell-intrinsic antiviral response compared to fibroblasts of her family members in similar conditions. The observed protective effect of IFN-γ *in vitro* however is artificial, since high dose IFN-γ will not be present 24 hours before an *in*

vivo infection to induce a preventive antiviral state. *In vivo* the type I IFN response is one of the first lines of defence against viral infections. The absence of this first innate immune defence in the STAT2-deficient patient creates a window of opportunity for viral pathogens to replicate and spread faster and further, hence the increased frequency and severity of viral infections in STAT2-deficient patients. However, once type II IFN is produced, it can aid in controlling the infection. This could explain why the viral susceptibility phenotype of STAT2-deficient patients appears to be milder than that of STAT1-deficient patients, in whom both type I and type II IFN signalling are disturbed [134].

Whether IFN-γ could be used in the treatment of STAT2-deficient patients is not clear at the moment. On the one hand, long term prophylactic treatment with IFN-γ has been used successfully and safely in patients with chronic granulomatous disease, a primary immunodeficiency disorder of phagocytes characterized by severe recurrent bacterial and fungal infections resulting from impaired killing of bacteria and fungi [167]. On the other hand, the life-threatening infectious episodes in the presented STAT2-deficient patients were accompanied by severe inflammation and immune dysregulation, even prompting investigations to exclude macrophage activation syndrome. Given the pro-inflammatory effects of IFN-γ and its role in macrophage activation [166], administration of IFN-γ during severe infectious episodes may improve antiviral immunity but may just as well lead to increased inflammation and clinical deterioration of the patient. Therefore, treatment with high dose IVIG together with supportive measures appear to be a safer option for life-threatening viral infections.

3.2 Difference in susceptibility to various viral pathogens in STAT2 deficiency

The susceptibility patterns observed in different PIDs demonstrate the interaction between different immune pathways and the virulence and immune evasion strategies of various viral pathogens. Just as in the first described kindred, the patients described in this work developed complicated disseminated measles following immunisation with the attenuated virus. This finding highlights the importance of type I IFN signalling and interferon-stimulated gene factor 3 (ISGF3)-complex formation in the

initial immune response against measles infections. However, while many of the common childhood viral illnesses had an unremarkable course in previously described STAT2-deficient individuals [75], the patients presented here also suffered from repeated and severe enterovirus infections ((+)ssRNA viruses), severe varicella infection and severe and prolonged Epstein-Barr virus (EBV) infection (both dsDNA viruses). Therefore, STAT2 deficiency in this second family appeared to be associated with increased susceptibility to a broader range of virus families than just (-)ssRNA viruses. It is not clear yet why some of the common childhood viral illnesses were more severe in the second kindred. Like in the patients from the first kindred with STAT2 deficiency, it cannot be concluded irrefutably that the STAT2 defect is complete and that this could be the reason for the more severe phenotype. Whether other genetic factors could be contributing to this finding, remains to be elucidated.

In contrast, the younger sibling of the second kindred experienced a relatively unremarkable RSV infection ((-)ssRNA virus) during the first year of life and did not require supportive therapy for this infection. This clinical observation correlates with the findings of the *in vitro* viral assay. While increased susceptibility of patient fibroblasts to *in vitro* infection with several (-)ssRNA viruses (measles virus (MeV), vesicular stomatitis virus (VSV), HPIV3) as well as a failure to signal through STAT2 in response to exogenous IFN-α was demonstrated, no increased susceptibility of patient fibroblast to RSV infection *in vitro* was detected compared to her mother and sibling. Additionally, pre-treatment with exogenous IFN-α partially reduced cell death in a similar manner during *in vitro* RSV infection in the patient, her mother and her sibling. Therefore, STAT2-deficient fibroblasts seemed to demonstrate similar behaviour during *in vitro* RSV infection as fibroblasts containing wild-type STAT2.

One potential explication for this finding of similar susceptibility to RSV infection could be that RSV preferentially inhibits type I IFN signalling in humans by downregulating the expression of STAT2 [168]. Additionally, many viruses can directly activate IRFs and lead to induction of ISGs [169]. In mice it has been demonstrated that RSV infection leads to upregulation of IRF1 and subsequent ISG induction independent of type I IFN signalling, through detection of the virus by intracellular RIG-I and IFIH1 receptors and subsequent activation of MAVS [170]. These combined

effects could explain why the fibroblasts of the patient, her mother and her sibling demonstrate a similar susceptibility to RSV infection *in vitro* in the untreated condition. Similar mechanisms are probably also involved in *in vivo* RSV infections in humans: recently a vital role for a MAVS-mediated IRF1/IFN-λ pathway was demonstrated in the antiviral response in human lung epithelial cells during RSV infection [170]. All these findings together with the observation of unremarkable RSV infection in a STAT2-deficient individual suggests that type I IFN signalling through the ISGF3-complex is not critically important in antiviral immunity during RSV infection.

It is not clear at the moment why patient fibroblasts experienced less cell death *in vitro* due to RSV infection after pretreatment with IFN-α, in a similar way as fibroblasts of her mother and sibling. Binding of IFN-α to IFNAR leads to phosphorylation of STAT1 and STAT2, and therefore formation and activation of mainly the ISGF3-complex but also weak activation of the GAF-complex [141]. Seeing how RSV decreases STAT2 expression in healthy individuals, one hypothesis could be that antiviral immunity against RSV is mostly GAF-dependent and ISGF3-independent, and that stimulation of IFNAR could sufficiently activate GAF to provide a similar protection in all family members.

4. Contribution of variants in *IFIH1*, *CECR1* and *STAT2* to common disease

In contrast with the possible monogenic nature of rare but severe early-onset disorders, diseases with later onset and higher prevalence in the population are thought to have a complex polygenic causation [5]. Less deleterious variants in genes associated with monogenic disorders could be contributing to these common forms of disease [5]. Less deleterious *IFIH1* variants, together with other genetic and environmental factors, could be contributing to the development of for example adult-onset SLE. Functional studies of the A946T *IFIH1* risk allele (rs1990760), associated with increased risk of developing SLE [171], type I diabetes mellitus [172] but also sIgAD [39] in GWAS, support this hypothesis. It is not clear whether increased base-line *IFIH1* expression is present in individuals carrying the risk allele, but the risk allele does seem to lead to an increased IFN-β response during enteroviral infection and

increased *IFIH1* expression following viral stimulation [173]. Therefore viral infection in individuals carrying the risk allele could lead to a higher and/or sustained IFN response capable of facilitating the onset of an inflammatory cascade that leads to autoimmune disease [173]. Several other common variants in or around the *IFIH1* gene have been associated with increased risk for autoimmune disease [173]. In the future functional studies might further explain how these polymorphisms could contribute to disease development by for example altering the function of regulation of the IFIH1 protein.

It seems also plausible that less deleterious *CECR1* variants could be contributing to for example development of vasculitis or strokes in early or late adulthood. No variants in or near *CECR1* have been identified as risk alleles for vasculitis or vasculopathy in GWAS, but the identification of *CECR1* mutations in Sneddon syndrome, an adult-onset autoinflammatory disorder characterized by intermittent fevers, livedoid rash, leg ulcerations and strokes, indicates that ADA2 deficiency can also present at a later age. Additionally the finding of the disease-causing p.Tyr453Cys *CECR1* mutation in heterozygous form in two brothers with late-onset lacunar strokes [69] suggests that variants in this gene leading to reduced plasma ADA2 activity could also contribute to more common vascular phenotypes in the general population. Likewise, while complete loss of STAT2 function causes increased and potentially fatal susceptibility to viral illness (especially to (-)ssRNA viruses such as measles), more common variants leading to reduced function of STAT2 could be involved in differences in viral susceptibility and disease severity within the general population.

5. Relevance of screening for alterations in the peripheral immune system

Advanced analysis of the peripheral immune system of patients and healthy individuals was performed, in order to identify alterations in the patient immune system compared to that of healthy age-matched individuals. Both cellular and humoral immune components were studied during the course of the project(s), and screening for immune cell alterations (i.e. changes in relative percentages of a limited set of immune cell subsets) in the examined patients compared to healthy age-matched controls was performed prior to genetic analysis. The latter was done for two

reasons: 1) to gain insight in disease mechanisms and pathways involved in disease pathogenesis by observing alterations in the patient's peripheral cellular immune system, and 2) to use the obtained information on immune cell alterations for the selection of candidate genetic variants.

Screening for immune cell alterations revealed important changes in immune cell subsets of certain patients, e.g. an immune phenotype compatible with active SLE in the patient with the *IFIH1* mutation or a defect in B cell differentiation and activation and a mild defect in T cell activation in the siblings with CID-like disease and ADA2 deficiency. However, the impact of using the immune cell screening results for the selection of candidate genetic variants and for gaining insight in disease pathogenesis was limited. In three out of four examined cases the immune cell alteration obtained from the screening did not contribute to the selection of a candidate genetic variant, but instead the candidate variants were identified by using the genetic analysis strategy outlined in the introduction. Also, the observed immune cell alterations often did not provide new information on disease pathogenesis but more often confirmed what was already known and described in the literature, as was the case for the patient with *IFIH1*-associated SLE. Several aspects are contributing to the limited utility of the proposed immune phenotype screening. First, this screening method only assesses alterations in a limited number of immune cell subsets, mostly of the adaptive immune system. However, the disease could be caused by a genetic defect presenting itself in other subsets of the cellular immune system that are not assessed or by a defect in innate immunity, as is the case in STAT2 deficiency where all patient immune cell subsets assessed were within normal range. Next, another difficulty lies in interpreting results of this type of analysis due to the large variability and heterogeneity in the (healthy) human immune system. Only large differences with healthy age-matched individuals stand out, with absence of an immune cell subset being one of the results easiest to interpret. Finally, an important problem is due to the influence of (mostly) environmental factors such as immunomodulatory medication or intercurrent infection on the appearance of an immune phenotype, which may be even more important than the underlying genetic defect.

There might be a difference between patients with disorders associated with a hyper or hypo immune state. Patients with a hyper immune state are almost always treated with immunosuppressive medication, which greatly influences and distorts the immune phenotype. Additionally, based on observations obtained in this work, the alterations in immune cell subsets tend to be less specific in patients with hyper immune states, rendering them less useful in the selection of candidate genetic variants. In contrast, in patients with a disorder due to a hypo immune state (i.e. PID) who normally do not receive immunosuppressive treatment, important defects in specific immune cell subsets can often be observed as was the case in the siblings with CID-like disease and ADA2 deficiency. In this case, the information on the defect in B cell differentiation/activation and the mild defect in T cell activation, together with the information on *in vitro* growth factor-like activity of ADA2 on monocytes and T helper cells found in the literature, was used for withholding the *CECR1* variant as a candidate.

A precise clinical description combined with a broad assessment of the function of the peripheral immune system, by examining both humoral (e.g. serum antibodies, cytokines) and cellular components (e.g. the main subsets of innate and adaptive immune cells [174]), should be part of the initial work-up of each examined case. When a candidate genetic variant is available for a patient, detailed investigations of humoral components or immune cell subsets that are altered or that may play a role in disease pathogenesis is important for establishing a cellular phenotype linked to the immune disease. Functional tests, for example by stimulating cell types with a variety of ligands and measuring the cell response, might be more reliable than a momentary assessment of a phenotype that is subjected to various modifying factors (e.g. immunomodulatory medication or intermittent infection). For example, in a large family with autoinflammation and neutrophilic dermatosis due to an autosomal inherited *MEFV* variant, involvement of mutated pyrin but not the NLRP3 inflammasome was demonstrated by an increased production of IL-1β by patient's monocytes compared to healthy controls in response to lipopolysacharide (LPS) but not to adenosine triphosphate (ATP) [15].

6. Clinical impact and contributions to patient care and well-being

The identification of the underlying genetic defect is often very important for these patients. Finally patients and their caregivers receive an explanation for their (child's) illness and for the substantial suffering they have endured over the course of their disease. Especially in families that have lost a child due to the illness, like the STAT2-family, this can provide some kind of closure. The psychological impact of finally being able to label their disease should also not be underestimated. The identification of a similar genetic defect in other patients/families over the world can provide insight into the disease prognosis and guide further treatment. For example, in the previously published family with STAT2 deficiency the affected individuals experienced a decrease in infection frequency and severity with increasing age, compatible with the development of their adaptive immune system, and the ones that survived childhood continued to lead a normal life [75].

Aside from the prognosis, the obtained insight in disease pathogenesis and the pathways involved can be used for the development of new treatments, and therapeutic experience of clinicians caring for other patients with similar genetic defects can be exchanged. For example, in ADA2 deficiency it became clear that the peri-HSCT stroke in the transplanted CID-sibling was in fact part of the phenotype, that allogeneic HSCT could be curative probably due to restoration of plasma ADA2 activity and that it is therefore absolutely necessary to perform this procedure for the younger brother as well to prevent additional strokes. Before the genetic defect was known, the parents of these ADA2-deficient children refused permission to perform a HSCT in the second affected child, seeing how this procedure almost resulted in the death of his older brother. The observation in the older sibling of the important inflammatory state pre- and peri-HSCT, intrinsic to ADA2 deficiency and the probable reason for all the complications that this child experienced peri-HSCT, will allow for the preventive use of biologicals in his younger brother to control inflammation before the start of the HSCT and hopefully prevent further complications. Even when no new treatment is available (or necessary) in the near future, the identification of the genetic defect opens up the possibility of genetic counselling and pre-implantation genetic

diagnosis for patients or (in case of recessive disorders) their carrier siblings who want to have children of their own.

CONCLUSION

The framework for genetic studies described in the introduction was successfully used to identify disease-causing variants in three different genes responsible for severe early-onset immune pathology in four unrelated families with very different clinical presentations. Together with numerous examples from the literature this demonstrates that genetic studies in single cases or families can be useful for implicating genes in severe early-onset immune pathology and for elucidating disease pathogenesis and pathways involved [14]. A precise clinical description combined with a broad assessment of the function of the peripheral immune system is essential for the genetic study of patients with disorders of immunity, and highlights the importance of collaborations between physicians and researchers with expertise in immune pathology.

1. Phenotypic heterogeneity associated with mutations in *IFIH1*, *CECR1* and *STAT2*

One of the main contributions of this work is that it extends the phenotypic spectrum associated with *IFIH1*-associated type I interferonopathies and ADA2 deficiency, and strengthens the data on STAT2 deficiency as the cause for increased susceptibility to viral illness.

Over the past 6-7 years the development and wide-spread use of NGS techniques have driven the discovery of genetic defects in a variety of human (immune) diseases, and the remarkable phenotypic heterogeneity associated with variants in a certain gene or even with the exact same genotype has become the rule rather than the exception. This phenotypic heterogeneity reflects the extreme diversity in human genetic variation that makes up a patient's genetic background, as well as the influence of the environment in the development of a phenotype. Mutations in the same gene can lead to a spectrum of diseases with variable severity, as can be seen in CAPS, a heterogeneous group of autoinflammatory disorders caused by gain-of-function mutations in *NLRP3* in which patients may present with three separate clinical conditions that form a disease-severity spectrum: familial cold autoinflammatory

syndrome (FCAS), Muckle-Wells syndrome (MWS) or neonatal-onset multisystem inflammatory disease (NOMID) [12]. Mutations in the same gene can also lead to disease manifestations in different organs. For example, gain-of-function mutations in *NOD2* (nucleotide-binding oligomerization domain-containing protein 2) are associated with Blau syndrome, an early-onset autoinflammatory disease characterised by articular, cutaneous and ocular non-caseating granulomatous inflammation, but *NOD2* mutations are also linked to susceptibility to Crohn's disease, a common polygenic inflammatory granulomatous bowel disease [175].

Gain-of-function mutations in *IFIH1* have been identified in a spectrum of disorders associated with elevated levels of type I IFN but with very different clinical presentations, such as SLE, AGS [135], HSP [42] and SMS [136]. STAT2 deficiency is responsible for increased susceptibility to viral infections, as demonstrated by the development of disseminated measles following the measles-mumps-rubella (MMR) vaccine in all described patients, but the severity of the phenotype can differ and in one family STAT2 deficiency was associated with mitochondrial dysfunction and subsequent neurological deterioration [133]. Finally, in ADA2 deficiency the entire spectrum of immunedysregulation is observed, ranging from an autoinflammatory syndrome (Castleman's-like disease) without immunodeficiency, to an autoinflammatory vasculitis/vasculopathy associated with hypogamma-globulinemia, to a CID-like presentation with autoimmunity, lymphoproliferation and subclinical inflammation. The last result nicely demonstrates the clinical/phenotypical overlap between autoimmunity, autoinflammation, immunodeficiency and even lymphoproliferation that can occur when a (mono)genetic defect affects an essential component of the immune system.

2. Treatment of ADA2 deficiency and STAT2 deficiency

Another important contribution of this work is that it provides vital scientific and clinical information on successful treatments of ADA2 deficiency, where treatment with IL-6 blockade was successful in controlling the immunological manifestations of Castleman's-like disease and allogeneic HSCT proved to be curative in one of the siblings with CID phenotype. Additionally, potential disease-related risks of HSCT in

ADA2 deficiency were described and several measures to prevent disease-related complications were suggested to guide future treatment of other patients. Several questions remain on the exact mechanisms through which disease control is achieved. ADA2 plasma activity was restored after allogeneic HSCT, and thus the restoration of normal plasma ADA2 levels could be responsible for the curative effect. Whether or not this translates into a restoration of ADA2 plasma activity in other tissues, whether this is even necessary and what the effect of absent ADA2 activity in other tissues on long-term prognosis might be, remains unclear. Disease control was also achieved through the use of anti-IL-6R mAbs (or TNFα blockade by other research groups [70, 164]). It is possible that controlling the inflammatory syndrome is sufficient to prevent vascular damage, however ADA2 might also exert growth factor-like activity on endothelial cells [69]. What the effect of absent ADA2 plasma activity on long-term prognosis might be, and whether patients might still be at risk for developing strokes/vasculopathy at a later age, remains unclear.

In STAT2 deficiency a good response to high dose IVIG was observed during (viral) infectious episodes associated with coagulopathy and severe immune dysregulation. The main hypothesis is that this response may in part be due to the known anti-inflammatory effect of high doses of IVIG [176], however, it is possible that passive immunization may also be adding to the observed effect. Observations in the three families with STAT2 deficiency show a decrease in disease frequency and severity with increasing age, possibly corresponding to the development of the adaptive immune system. However, one sibling did succumb to an infection with a (probably viral) agent, indicating that patients may still be at risk for fatal viral disease beyond early childhood. Therefore, the question remains whether STAT2-deficient patients should be treated with Ig replacement therapy during childhood. A conservative treatment approach was taken in the three described families, with a low threshold for high dose IVIG treatment for the patient described in this work. This reflects the relatively mild nature of this PID, however more information on clinical course and prognosis of additional STAT2-deficient patients is necessary to guide future treatment.

FUTURE RESEARCH

Disease-causing variants leading to a wide spectrum of clinical manifestations were identified in three genes: *IFIH1, CECR1* and *STAT2*. Many questions remain on disease pathogenesis, modifying factors in disease development, the possible contributions of variants in *IFIH1*, *CECR1* and *STAT2* to common disease forms and potential treatments of the immunological diseases described in this work.

Disease mechanisms through which variants in *IFIH1* and *STAT2* lead to respectively increased levels of type I IFN and loss of type I IFN signalling, have been identified and described by other research groups [75, 135]. It is still not clear though, how increased levels of IFN can give rise to such a wide spectrum of clinical manifestation in individuals with *IFIH1* mutations, ranging from an autoinflammatory encephalopathy to a prototypic autoimmune disease to asymptomatic individuals. The identification of modifying genetic or environmental factors that drive disease towards a certain clinical presentation, will be a major challenge. As *IFIH1* mutations are expected to be identified more frequently in other individuals, the study of this cohort of *IFIH1* mutation-positive individuals by for example GWAS to determine the burden of known autoimmune disease risk alleles, may provide insight into the interplay between this 'Mendelian' mutation and modifying genetic factors.

Many questions on the disease pathogenesis of ADA2 deficiency remain unanswered as well. Potentially contributing mechanisms could be proinflammatory polarization of macrophages and disturbed endothelial integrity due to loss of ADA2 [69]. However, how ADA2 deficiency could lead to increased production of IL-6, what the role of IL-6 might be in inflammation and vascular damage associated with ADA2 deficiency and whether IL-6 and its effects are present in all ADA2-deficient patients, remains unclear. The lack of a *CECR1* ortholog in commonly used laboratory animals such as mouse or rat and therefore the absence of such animal models of ADA2 deficiency renders this questions all the more difficult to answer. Although the main feature of systemic inflammation has been found in all ADA2-deficient patients described so far, a spectrum of clinical manifestations can be found and no information is available yet

on possible modifying factors that could shape the clinical presentation of ADA2 deficiency.

The continued search for individuals with disease-causing variants in *IFIH1, CECR1* and *STAT2* can provide additional information on potential clinical disease manifestations and severity, response to different treatments and prognosis. For example, identification of additional patients with STAT2 deficiency can help clarify the question of long-term prognosis and the risk of contracting life-threatening viral disease at a later age. This can in turn guide further treatment of STAT2-deficient patients. Similarly, in ADA2 deficiency long-term follow-up of ADA2-deficient patients who received allogeneic HSCT or whose disease was controlled by biologicals (without restoration of ADA2 plasma activity), is necessary to gain insight into long-term effects of the different therapeutic options and to guide future treatment. Several treatments targeting the type I IFN signalling pathway such as the anti-IFNAR mAb anifrolumab, the JAK1/3-inhibitor tofacitinib or the JAK1/2-inhibitor baricitinib are being tested in clinical trials in patients with SLE or type I interferonopathies such as CANDLE and SAVI. Positive outcomes in these trials could open up the possibility of targeted therapies in (IFIH1-associated) type I interferonopathies.

Similarly, (additional) common variants in or near *IFIH1*, *CECR1* and *STAT2* may be identified that could potentially contribute to more common forms of immune disease. In the future functional studies might further explain how these polymorphisms could contribute to disease development by for example altering the function or regulation of the respective proteins. Finally, many other genes may be responsible for or could be contributing to the development of certain immune phenotypes. For example, other underlying genetic defect(s) in iMCD, a genetically heterogeneous group of disorders, still need to be elucidated. Likewise, the underlying genetic defects in most of the different vasculitis forms have not been identified yet. Many patients with unique and potentially Mendelian phenotypes who need a genetic diagnosis and targeted treatment, are still available for study, and genetic studies on these patients can increase our understanding of the physiology and pathology of the human immune system.

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European Society for Immunodeficiency (ESID), Prague, 29 October - 1 November 2014.

- *Novel Compound Heterozygous Mutation in STAT2 in Two Patients Suffering from Severe Viral Infections.* Lien Van Eyck, Glynis Frans, Leen Moens, D.Pombal, Isabelle Meyts, Adrian Liston. (Poster presentation)
- *HSCT Rescues the Immunological and Vascular Phenotype of ADA2-Deficiency.* Lien Van Eyck, Michael S.Hershfield, Diana Pombal, Susan J.Kelly, Nancy J. Ganson, Leen Moens, Glynis Frans, Heidi Schaballie, Gert DeHertogh, James Dooley, Xavier Bossuyt, Carine Wouters, Adrian Liston, Isabelle Meyts. (Poster presentation)
- *Severe Defect in Regulatory T Cell Homeostasis in a Murine Model for Familial Hemophagocytic Lymphohistiocytosis.* Stéphanie Humblet-Baron, Simon Bornschein, Lien Van Eyck, Carine Wouters, Frans Baron, Patrick Matthys, Adrian Liston. (Poster presentation)

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